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Nicotinic Acetylcholine Receptors in Experimental Models of Parkinson's Disease and Levodopa-Induced Dyskinesia: Focus on α5 Subunit-Containing Receptors

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Contents

Abstract

No cure exists for Parkinson's disease (PD), a disease marked by the degeneration of dopaminergic neurons of the substantia nigra pars compacta (SNC), a loss of dopamine in the dorsal striatum, and resulting motor symptoms. Furthermore, treatment of PD with levodopa is often complicated by abnormal involuntary movements (levodopa-induced dyskinesia, LID). Novel treatment options for PD and LID are thus greatly needed. Nicotinic acetylcholine receptors represent one possible novel treatment target, given the complex control they exert over dopaminergic neurotransmission, protective effects of smoking against PD, and extensive preclinical evidence of neuroprotective and antidyskinetic effects by nicotinic receptor ligands. Nicotinic receptor subtypes essential for nigrostriatal dopaminergic neurotransmission include those containing the α5 subunit, which have not been previously studied in the context of PD.

In this thesis, further preclinical investigations of the role of nicotinic receptors in PD and LID were carried out. An extensive *in vivo* and *ex vivo* characterization of the role of α5-containing receptors in mouse models of PD was performed. The effects on LID by chronic nicotine treatment in drinking water and other drug treatments were studied *in vivo* utilizing mouse models of both moderate and severe PD and LID. The mechanisms of action underlying LID and the antidyskinetic effects of nicotine were studied by *ex vivo* measurements of striatal dopamine release and corticostriatal brain-derived neurotrophic factor (BDNF). In parallel, methods for stereotactic surgery and postoperative care were significantly improved.

Mice lacking α5-containing nicotinic receptors were found to be less susceptible to unilateral nigrostriatal neurodegeneration, the resulting interhemispheric motor imbalance, and LID. Striatal dopamine uptake measurements suggested reduced dopamine transporter function as a possible mechanism of neuroprotection. Nicotine was found to inhibit LID, with findings suggesting a role for α6β2* nicotinic receptors. However, neither nicotinic receptor agonists nor the clinically used drug amantadine alleviated severe LID associated with near-total dopaminergic denervation. The findings also confirmed a correlation between striatal BDNF and LID. The present findings suggest the potential usability of α5-containing nicotinic receptors as a drug target against PD and LID. The findings also confirm the preclinical potential of nicotine as an antidyskinetic drug while suggesting limited efficacy in advanced PD. In addition, the findings expand previous knowledge on the possible mechanisms of LID and the antidyskinetic effects of nicotine.

Tiivistelmä

Parkinsonin taudin motoriset oireet aiheutuvat mustatumakkeen dopamiinihermosolujen rappeutumisesta ja dopamiinin puutoksesta aivojuoviossa. Sairaudelle ei ole löydetty parantavaa hoitoa. Lisäksi tahdottomat liikkeet (dyskinesia) vaikeuttavat usein sairauden hoitoa levodopa-lääkeaineella. Uusia mahdollisuuksia Parkinsonin taudin ja dyskinesian hoitoon siis tarvitaan. Asetyylikoliinin nikotiinireseptorit voisivat tarjota yhden mahdollisen uuden lääkevaikutuskohteen, sillä ne säätelevät aivojen dopamiinijärjestelmää, tupakoinnin tiedetään suojaavan Parkinsonin taudilta, ja laaja prekliininen näyttö viittaa nikotiinireseptoriligandeilla olevan hermosoluja suojaavia ja dyskinesiaa lieventäviä vaikutuksia. Dopaminergisen hermovälityksen kannalta oleellisiin nikotiinireseptorialatyyppeihin kuuluvat α5-alayksikön sisältävät reseptorit, joiden yhteyttä Parkinsonin tautiin ei aiemmin ole tutkittu.

Tässä väitöskirjassa tutkittiin nikotiinireseptorien merkitystä Parkinsonin taudissa ja levodopan aiheuttamassa dyskinesiassa. α5-alayksikön sisältävien nikotiinireseptorien merkitystä Parkinsonin taudin ja dyskinesian hiirimalleissa tutkittiin kattavin *in vivo*- ja *ex vivo*-kokein. Juomaveden kautta annetun pitkäkestoisen nikotiinikäsittelyn ja muiden lääkehoitojen vaikutuksia tutkittiin sekä kohtalaisen että pitkälle edenneen Parkinsonin taudin ja dyskinesian hiirimalleissa. Dyskinesian ja nikotiinin dyskinesiaa lievittävien vaikutusten mekanismeja tutkittiin mittaamalla *ex vivo* dopamiinin vapautumista aivojuoviosta sekä aivoperäisen hermokasvutekijän (BDNF) pitoisuuksia aivoissa. Samanaikaisesti stereotaktisten leikkausten ja leikkauksen jälkeisen hoidon menetelmiä kehitettiin merkittävästi.

α5-poistogeenisten hiirten havaittiin olevan vähemmän herkkiä dopamiinihermojen toispuoleiselle tuhoutumiselle, siitä johtuvalle aivopuoliskojen motoriselle epätasapainolle sekä levodopan aiheuttamalle dyskinesialle. Dopamiinin soluunottoa aivojuoviossa mitanneiden kokeiden perusteella dopamiinin kuljettajaproteiinin heikentynyt toiminta saattoi olla yksi hermosoluja suojaava mekanismi. Nikotiinin todettiin lievittävän levodopan aiheuttamaa dyskinesiaa, ja α6β2* nikotiinireseptorit voivat olla tärkeitä tämän vaikutuksen välittäjiä. Nikotiinireseptorien agonistit tai kliinisesti käytetty lääkeaine amantadiini eivät kuitenkaan lievittäneet vakavaa, lähes täydelliseen dopamiinihermokatoon liittyvää dyskinesiaa. Tutkimuslöydökset myös vahvistivat yhteyden BDNF:n ja levodopan aiheuttaman dyskinesian välillä. Tutkimuksen tulokset viittaavat siihen, että α5-alayksikön sisältävät nikotiinireseptorit voisivat olla mahdollinen uusi vaikutuskohde Parkinsonin taudin hoidossa. Löydökset myös vahvistavat nikotiinin olevan mahdollinen levodopan aiheuttaman dyskinesian hoitomuoto, mutta viittaavat siihen, että hoitovaste pitkälle edenneessä Parkinsonin taudissa voi olla heikko. Lisäksi tutkimus laajentaa aiempaa tietämystä dyskinesian ja nikotiinin dyskinesiaa lievittävien vaikutusten mahdollisista mekanismeista.

Original publications

This thesis is based on the following original publications:

I Leino, S., Koski, S.K., Hänninen, R., Tapanainen, T., Rannanpää, S., Salminen, O. (2018). Attenuated dopaminergic neurodegeneration and motor dysfunction in hemiparkinsonian mice lacking the α5 nicotinic acetylcholine receptor subunit. Neuropharmacology 138: 371–380

II Leino, S., Koski, S.K., Rannanpää, S., Salminen, O. (2018). Effects of antidyskinetic nicotine treatment on dopamine release in dorsal and ventral striatum. Neuroscience Letters 672: 40–45

III Leino, S., Kohtala, S., Rantamäki, T., Koski, S.K., Rannanpää, S., Salminen, O. (2018). Dyskinesia and brain-derived neurotrophic factor levels after long-term levodopa and nicotinic receptor agonist treatments in female mice with near-total unilateral dopaminergic denervation. BMC Neuroscience 19: 77

IV Koski, S.K., Leino, S., Rannanpää, S., Salminen, O. (2019). Implementation of improved postoperative care decreases the mortality rate of operated mice after an abundant 6-hydroxydopamine lesion of nigrostriatal dopaminergic neurons. Scandinavian Journal of Laboratory Animal Science 45: 1

The publications are referred to in the text by the above roman numerals. Reprints were made with the permission of the copyright holders.

Abbreviations

5-HT	5-hydroxytryptamine (serotonin)
6-OHDA	6-hydroxydopamine
AADC	aromatic-L-amino-acid decarboxylase
ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
cAMP	cyclic adenosine monophosphate
ChI	cholinergic interneuron
CNS	central nervous system
CtxMII	α-conotoxin MII
D1R	dopamine D1 receptor
D2R	dopamine D2 receptor
D3R	dopamine D3 receptor
DARPP-32	dopamine and cAMP-regulated phosphoprotein, 32 kDa
DAT	dopamine transporter
DBS	deep brain stimulation
dMSN	direct pathway medium spiny neuron
DOPAC	3,4-dihydroxyphenylacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
GABA	γ-aminobutyric acid
GPe	external segment of the globus pallidus
GPi	internal segment of the globus pallidus
HPLC	high-performance liquid chromatography
HVA	homovanillic acid
IEG	immediate early gene
iMSN	indirect pathway medium spiny neuron
LID	levodopa-induced dyskinesia
LTD	long-term depression
LTP	long-term potentiation
mAChR	muscarinic acetylcholine receptor
MAO	monoamine oxidase
MFB	medial forebrain bundle
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP+	1-methyl-4-phenylpyridinium
mRNA	messenger ribonucleic acid
MSN	medium spiny neuron
nAChR	nicotinic acetylcholine receptor
PBS	phosphate-buffered saline
PD	Parkinson's disease

pDAT	phospho (T53)-dopamine transporter
PET	positron emission tomography
PFA	paraformaldehyde
PFC	prefrontal cortex
PKA	protein kinase A
qPCR	quantitative polymerase chain reaction
RM-ANOVA	repeated measures analysis of variance
SEM	standard error of the mean
SERT	serotonin transporter
SN	substantia nigra
SNC	substantia nigra pars compacta
SNCD	dorsal tier of the substantia nigra pars compacta
SNCM	medial cluster of the substantia nigra pars compacta
SNR	substantia nigra pars reticulata
STN	subthalamic nucleus
TH	tyrosine hydroxylase
TTBS	Tween-20 in Tris-buffered saline
VTA	ventral tegmental area

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease affecting the dopaminergic neurons of the brain nigrostriatal pathway that leads, among other symptoms, to progressively worsening difficulties in movement (Jankovic, 2008). As no treatment affecting the progression of the neurodegeneration is available, current treatments for Parkinson's disease can only alleviate its symptoms (Fox et al., 2018). While symptomatic treatment with the dopamine precursor levodopa is usually effective, long-term treatment is often associated with serious adverse effects (Aquino and Fox, 2015). Among these adverse effects are abnormal involuntary movements, termed levodopa-induced dyskinesia (LID), which affect in some form nearly every patient with long enough treatment. Treatment options for LID with strong evidence for efficacy are sparse, only including pharmacological treatment with amantadine and invasive deep-brain stimulation (Fox et al., 2018). Novel options for the treatment of PD and LID are thus sorely needed.

Such novel treatment options have been sought among many other avenues by investigating the role of nicotinic acetylcholine receptors (nAChRs) in Parkinson's disease. nAChRs are receptors for the endogenous neurotransmitter acetylcholine and are widely expressed throughout the nervous system and the whole body as numerous different subtypes (Albuquerque et al., 2009; Millar and Gotti, 2009). Exogenous ligands of nicotinic receptors include the prototypical and eponymous non-selective agonist nicotine, the main psychoactive component of tobacco, as well as many toxins and investigative drugs. In the central nervous system (CNS) nicotinic receptors act primarily as neuromodulators, regulating the activity of many neurotransmitter systems and brain areas, including the dopaminergic nigrostriatal pathway (Dani and Bertrand, 2007; Quik and Wonnacott, 2011). Accumulating epidemiological and preclinical evidence suggests potential for nAChRs as drug targets for PD, with such findings as decreased risk of PD in users of tobacco products, dopaminergic neuroprotection by nAChR agonists in cellular and animal models of neurodegeneration, and alleviation of LID by various nAChR ligands (Quik and Wonnacott, 2011).

The studies comprising this thesis utilized mouse models of PD and LID, and aimed at further investigation of the role of nAChRs in PD and LID as well as their potential usability as targets in the treatment of the disease. Particular focus was afforded to nAChRs containing the α5 subunit, which have an important role in nigrostriatal dopaminergic neurotransmission (Salminen et al., 2004; Exley et al., 2012) but have not been previously studied in the context of PD. In the first part of this thesis, the relevant literature will be reviewed, with particular attention on levodopa-induced dyskinesia and on nicotinic receptors in Parkinson's disease. The subsequent parts contain full descriptions of the methods and results of the present studies. Finally, discussion will be presented both on methodological issues, including a brief description of the various animal models of Parkinson's disease, and on the results obtained and their implications.

2. Review of the literature

2.1. Basal ganglia and the nigrostriatal dopaminergic pathway

2.1.1. Anatomy and neurocircuitry of the basal ganglia

The cardinal motor symptoms of Parkinson's disease stem primarily from a selective progressive death of neurons in the midbrain region of substantia nigra (SN), in particular its subdivision substantia nigra pars compacta (SNC). This brain area is located within the basal ganglia, a group of subcortical nuclei at the base of the forebrain. The basal ganglia form an interconnected network of brain areas with many suggested and debated functions, including the selection of actions (Cisek and Kalaska, 2010) as well as control over movement speed and size, movement error correction, motor learning, and recall of motor skills (Turner and Desmurget, 2010). In brief, it is thought that motor information from the cortex and the thalamus arrives at the dorsal striatum (caudate-putamen in humans), where it is integrated with inputs from other basal ganglia nuclei, including dopaminergic input from the SNC, and then transmitted to output nuclei of the basal ganglia and from thereon to targets of basal ganglia output (Albin et al., 1989; Smith et al., 1998; Bolam et al., 2000; Gerfen and Surmeier, 2011). The discussion in this thesis will mainly be confined to basal ganglia regions primarily related to movement control (e.g., the dorsal striatum and the SN). Regions that are primarily associated with limbic functions and less affected in PD (e.g., the ventral striatum and the ventral tegmental area; Joel and Weiner, 2000) will be mostly ignored.

See Figure 1 for a schematic representation of the organization of the basal ganglia. The striatum is the main input nucleus of the basal ganglia and receives excitatory glutamatergic projections from the cortex, with essentially all sensory, motor and associative cortical areas sending somatotopically organized projections to the dorsal striatum (Smith et al., 1998; Bolam et al., 2000). In addition, the dorsal striatum receives glutamatergic projections from the thalamus (Doig et al., 2010). The vast majority of all striatal neurons (up to 95 % in rodents) consist of medium spiny neurons (MSN), which are inhibitory neurons utilizing γ-aminobutyric acid (GABA) that receive the corticostriatal and thalamostriatal inputs along with inputs from the midbrain such as from the SNC (Smith et al., 1998; Bolam et al., 2000). In turn, MSNs send striatal output to the two output nuclei of the basal ganglia, substantia nigra pars reticulata (SNR) and the internal segment of the globus pallidus (GPi), via two pathways (Smith et al., 1998; Bolam et al., 2000). Although evidence challenging strict segregation exists (e.g., Cazorla et al., 2014), these two pathways are regarded as largely separate, and termed the direct and indirect pathways according to the route the striatal information takes to arrive at the output nuclei (Figure 1). In brief, the direct pathway consists of direct inhibitory projections from the striatum to the SNR and the GPi. In contrast, the indirect pathway consists of inhibitory projections from the striatum to the external segment of the globus pallidus (GPe), followed by inhibitory projections from the GPe to not only the SNR and GPi but also to the subthalamic nucleus (STN), which

in turn sends excitatory projections to the SNR and the GPi. As the output nuclei SNR and GPi are also GABAergic and inhibitory, activation of the inhibitory direct pathway results in disinhibition of the target structures of basal ganglia output, while activation of the indirect pathway provides excitation to the output nuclei, resulting in inhibition of their targets (Smith et al., 1998; Bolam et al., 2000). Besides the above simplified description, many additional projections, feedback loops and parallel interconnections are thought to exist, including excitatory cortical input to the STN, excitatory projections from the STN to the GPe, and inhibitory "bridging" collaterals from striatal direct pathway MSNs to the GPe (Smith et al., 1998; Gerfen and Surmeier, 2011; Cazorla et al., 2014; Ko et al., 2014).

The target structures of the basal ganglia output nuclei SNR and GPi include subcortical "premotor" regions (e.g., the superior colliculus, the pedunculopontine nucleus, and the

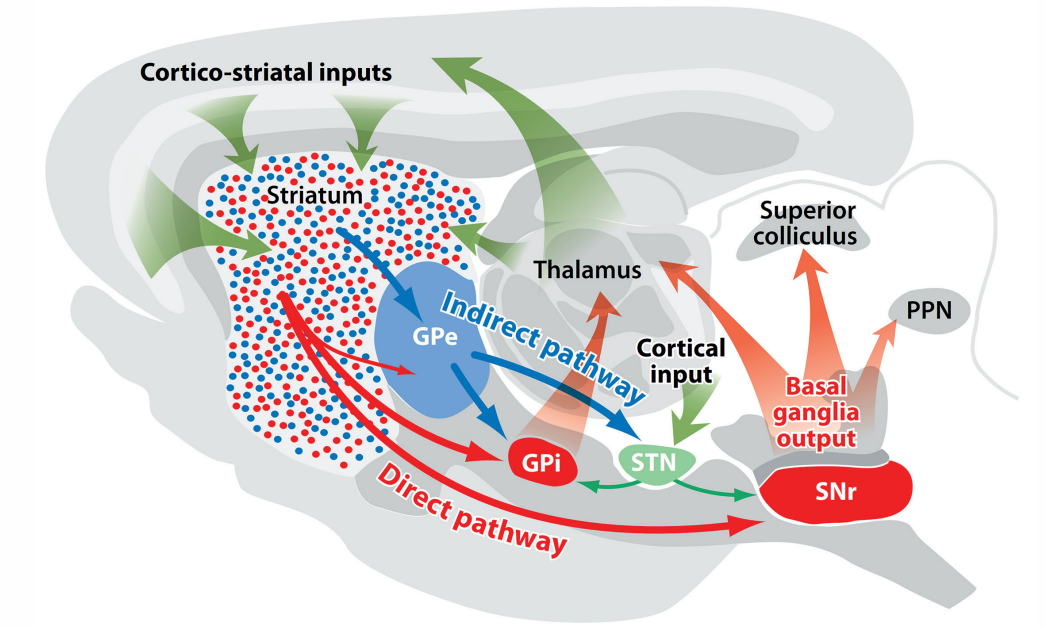

Figure 1. Basal ganglia organization shown in the mouse brain. The striatum receives input from the cortex and the thalamus. The direct pathway projects directly to the output nuclei, the internal segment of the globus pallidus (GPi) and the substantia nigra pars reticulata (SNr). The indirect pathway projects first to the external segment of the globus pallidus (GPe) and from there both to the output nuclei and to the subthalamic nucleus (STN). The STN in turn sends projections to the output nuclei. Finally, the GPi and the SNR send projections to various target regions. Green arrows represent excitatory glutamatergic projections, other arrows represent inhibitory GABAergic connections. The final outcome of direct and indirect pathway activation is disinhibition and inhibition of the target regions, respectively. Dopaminergic pathways are not shown and many connections are omitted. PPN = pedunculopontine nucleus. Republished with permission from Annual Reviews: Annual Reviews of Neuroscience, Gerfen and Surmeier (2011), © Annual Reviews 2011

reticular formation) and, most importantly, the thalamus with its projections to motor and premotor cortical areas (Bolam et al., 2000). The classical model of basal ganglia neurotransmission suggests that under resting conditions the basal ganglia network provides tonic inhibition of the target structures, with signals originating from the cortex first eliciting disinhibition of the target structures, mediated by the direct pathway and associated with behaviors such as movement, while subsequent inhibition of the target structures mediated by the indirect pathway acts to terminate the behavior (Albin et al., 1989; Smith et al., 1998; Bolam et al., 2000). Further elaborations suggest that the striatum may act as an integrator of competing input from different motor systems, with a particular behavior selected, and others inhibited, by coordinated activity of the two pathways (Cisek and Kalaska, 2010; Gerfen and Surmeier, 2011). Moreover, recent evidence suggests that the two pathways are likely to overlap and intertwine, with synaptic plasticity phenomena perhaps determining whether MSN activity encodes action initiation or inhibition (Calabresi et al., 2014). Based on findings challenging the classical model, such as no impairment of movement by lesions of the GP, it has even been suggested that the basal ganglia do not in fact have an essential role in moment-to-moment movement control, but rather serve higher functions such as motor learning (Obeso et al., 2017).

2.1.2. Dopaminergic modulation of basal ganglia neurotransmission

Dopamine (3,4-dihydroxyphenylethylamine) is a catecholamine compound found particularly in brain areas involved in motor control such as the SN and the striatum (Joel and Weiner, 2000; Björklund and Dunnett, 2007). Neurons within the SNC send projections ending in dopamine-releasing synaptic terminals in the dorsal striatum (Joel and Weiner, 2000; Björklund and Dunnett, 2007). These neurons and their projections are often termed the nigrostriatal pathway. Figure 2 illustrates the major ascending dopaminergic pathways of the midbrain, including not only the nigrostriatal pathway but two other major ascending dopaminergic systems. The latter arise mainly from the ventral tegmental area (VTA) and consist of the mesolimbic pathway, projecting to the ventral striatum (nucleus accumbens and olfactory tubercle), and the mesocortical pathway (Joel and Weiner, 2000; Björklund and Dunnett, 2007). Note, however, that the common concept of a strict division of these dopaminergic projections is an oversimplification; particularly in the primate brain, some SNC dopaminergic neurons send projections also to limbic and cortical areas and, vice versa, the dorsal striatum receives innervation also from such areas as the VTA (Björklund and Dunnett, 2007). Projections from various SNC subdivisions are also at least partially segregated according to striatal compartments (patches and matrix; Joel and Weiner, 2000; Matsuda et al., 2009). Further subdivisions of brain areas such as the SNC have also been described in various species using various nomenclature (see e.g., Björklund and Dunnett, 2007; Fu et al., 2012). Finally, note that midbrain dopaminergic neurons innervate not only the striatum and the cortex, but also many other basal ganglia nuclei (Björklund and Dunnett, 2007). All in all, midbrain dopaminergic systems demonstrate a complex organization with numerous components and significant species differences (Joel and Weiner, 2000; Björklund and Dunnett, 2007).

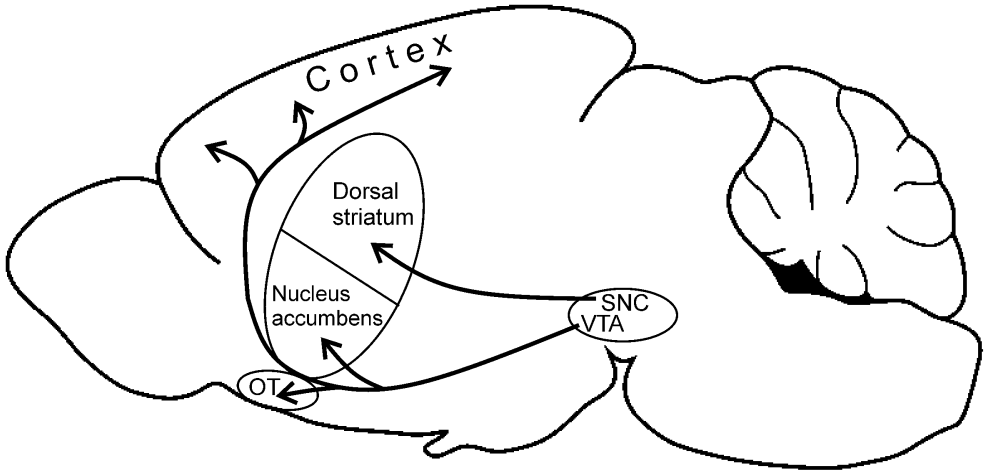

Figure 2: Main dopaminergic areas of the ventral midbrain and ascending dopaminergic pathways, shown in the mouse brain. The nigrostriatal pathway projects from the substantia nigra pars compacta (SNC) to the dorsal striatum. The mesolimbic pathway projects from the ventral tegmental area (VTA) to the ventral striatum, including the nucleus accumbens and the olfactory tubercle (OT). The mesocortical pathway projects from the VTA to cortical areas. Anatomical accuracy approximate only. Information source: Björklund and Dunnett (2007)

Midbrain dopaminergic neurons exhibit both regular spontaneous single spike firing (tonic activity) and burst firing (phasic activity); the latter occurs in response to novel external stimuli with behavioral significance, particularly unpredicted rewarding stimuli, and has been suggested to contribute to the neural basis of reward prediction and learning (Schultz, 2007). Of most relevance to Parkinson's disease, however, is the critical role that nigrostriatal dopaminergic neurons have in modulating basal ganglia activity underlying movement control. In the dorsal striatum, nigrostriatal dopaminergic neurons form dense, widespread, and highly overlapping axonal arborizations, where in rodents a single neuron can cover up to 5 % of the entire target brain area, communicating with tens of thousands of striatal neurons, while one striatal MSN can in turn be influenced by up to 200 dopaminergic neurons (Matsuda et al., 2009). The main form of dopaminergic control of striatal activity is believed to be the modulation of the corticostriatal and possibly also thalamostriatal glutamatergic input to the MSN projection neurons (Surmeier et al., 2007; Gerfen and Surmeier, 2011). Importantly for dopaminergic modulation, direct pathway MSNs (dMSN) and indirect pathway MSNs (iMSN) differ not only in their efferent projections but their expression of dopamine receptors. More specifically, dMSNs express dopamine D1 receptors (D1R) while iMSNs express dopamine D2 receptors (D2R; Surmeier et al., 2007; Gerfen and Surmeier, 2011).

Through numerous molecular mechanisms, dopamine activates striatonigral dMSNs through D1 receptors, enhancing D1R MSN responsiveness to coordinated glutamatergic input, while inhibiting striatopallidal iMSNs through D2 receptors, reducing presynaptic glutamate release and D2R MSN responsiveness to glutamate (Surmeier et al., 2007; Gerfen and Surmeier, 2011). These mechanisms include the modulation of such phenomena as Ca^{2+}, Na^{+} and K^{+} ion currents as well as glutamate receptor function and trafficking (Surmeier et al., 2007; Gerfen and Surmeier, 2011). In addition, dopamine exerts a modulating effect on activity-dependent plasticity of striatal glutamatergic synapses, possibly mediated in part via interneurons, with the magnitude and direction of plasticity (depression or potentiation) determined in part by the presence of postsynaptic D1 vs. D2 receptors as well as dopamine release kinetics (Surmeier et al., 2007; Wickens, 2009; Gerfen and Surmeier, 2011). On the intracellular level, dopamine receptor signaling is very complex. Very briefly, the main signaling pathway for D1R is the $G\alpha_{s/olf}$- mediated activation of adenylate cyclase, resulting in cyclic adenosine monophosphate (cAMP) elevation and activation of protein kinase A (PKA), which then has numerous direct and indirect targets such as the dopamine and cAMP-regulated phosphoprotein, 32 kDa (DARPP-32), the extracellular signal-regulated kinase (ERK), and glutamatergic ion channels (Beaulieu et al., 2015). The main signaling pathway for D2R, in contrast, is the $G\alpha_{i/o}$-mediated inhibition of adenylate cyclase; both receptor types also have numerous other downstream targets (Beaulieu et al., 2015).

The dopaminergic modulation of corticostriatal neurotransmission is in turn subject to complex modulation by numerous other projections and neurotransmitter systems. Striatal dopamine release is presynaptically modulated by D2 autoreceptors as well as numerous heteroreceptors such as glutamatergic, GABAergic, and nicotinic acetylcholine receptors (Zhang and Sulzer, 2012). In the SNC, dopaminergic neuron firing is regulated both by inputs from within the basal ganglia, including glutamatergic input from the STN and GABAergic input from the striatum, globus pallidus, and SNR, as well as by inputs from outside the basal ganglia, including motor and somatosensory cortical areas (Lee and Tepper, 2009; Watabe-Uchida et al., 2012). Finally, SNC dopaminergic neurons can also release dopamine from their somatodendritic areas, thus modulating both their own activity through autoreceptors and GABAergic interneurons as well as the activity of the basal ganglia output neurons of the neighboring SNR (Lee and Tepper, 2009; Rice and Patel, 2015).

The exact nature of how the dopaminergic modulation of corticostriatal neurotransmission relates to the postulated action selection function of the basal ganglia remains unclear. One suggestion is that the burst activity exhibited by dopaminergic neurons in response to rewarding events may be translated to action selection by modulation of the corticostriatal glutamatergic signals and striatal output (Gerfen and Surmeier, 2011). Other researchers believe that it is the regular tonic release of dopamine that is essential for normal basal ganglia function, perhaps by regulating short-term synaptic plasticity (Obeso et al., 2017). Nevertheless, the dramatic impact of the loss of striatal dopamine on movement initiation

and control, as exhibited for instance in Parkinson's disease, demonstrates the crucial role of the dopaminergic modulation of the basal ganglia. In particular, imbalances between direct and indirect pathways may underlie the symptoms of many movement disorders (see 2.2.2. and Figure 4).

2.1.3. Striatal interneurons and non-dopaminergic neurotransmission

Although MSNs comprise the vast majority of striatal neurons, various interneurons also have significant roles in striatal neurotransmission. These neurons can be broadly divided into cholinergic interneurons and two types of GABAergic interneurons (Gittis and Kreitzer, 2012). The GABAergic interneurons, including fast-spiking interneurons and persistent and low-threshold spiking interneurons, comprise 3–4 % of striatal neurons and exert a complex regulation of striatal activity, are in turn modulated by nigrostriatal and corticostriatal inputs, and are thought to contribute to the pathophysiology of several basal ganglia-related movement disorders such as PD (Gittis and Kreitzer, 2012). The remaining 1–3 % of striatal neurons are cholinergic interneurons (ChI), which exhibit widespread intrastriatal connectivity, exert a modulatory control on most striatal neurotransmission (Aosaki et al., 1994; Oldenburg and Ding, 2011), and are also thought to be involved in a number of movement disorders such as PD and LID (see 2.2.2. and 2.3.2.).

The tonically active ChIs are thought to serve as integrators of dopaminergic and cholinergic influences in the striatum due to showing pauses in the tonic firing in response to conditioned stimuli (Aosaki et al., 1994). More recently, ChIs have been suggested to serve as an integral part of a thalamostriatal circuit regulating corticostriatal inputs to MSNs and underlying the cessation of behaviors in response to salient stimuli (Ding et al., 2010). Acetylcholine released by ChIs modulates MSN activity directly through metabotropic M1 and M4 muscarinic receptors (mAChR) as well as indirectly through various mAChRs expressed on corticostriatal glutamate terminals, GABAergic interneurons, and ChIs themselves (Oldenburg and Ding, 2011). In addition, acetylcholine modulates the striatal release of most neurotransmitters via presynaptic nAChRs; see 2.4.2. for a detailed discussion on nAChR-mediated neuromodulation in the basal ganglia. Thus, striatal acetylcholine acting through mAChRs and nAChRs serves as a complex modulator of glutamatergic neurotransmission, MSN excitability, and striatal output. Note that while ChIs were long considered the sole source of striatal acetylcholine, recently also direct cholinergic projections from the brainstem to the striatum have been described (Dautan et al., 2014).

In addition to those described in the above sections, still a number of other, less well characterized striatal inputs exist, including noradrenergic projections from the pedunculopontine nucleus and the locus coeruleus as well as glutamatergic projections from the amygdala (Parent et al., 1983; Smith et al., 1998). Finally, worth specific mentioning are serotonergic projections from the medial and dorsal raphe nuclei that innervate both the midbrain dopaminergic regions and the striatum (Alex and Pehek, 2007). Numerous

serotonin receptor subtypes participate in the regulation of striatal dopamine release (Alex and Pehek, 2007), and striatal serotonin terminals are also highly relevant for abnormal presynaptic handling of levodopa associated with LID (see 2.3.2.).

2.2. Parkinson's disease and dopaminergic neurodegeneration

2.2.1. Clinical features and epidemiology of Parkinson's disease

The motor disorder now known as Parkinson's disease was first systematically described in western literature by James Parkinson in his seminal 1817 monograph "An Essay on the Shaking palsy" (reprinted as Parkinson, 2002). The classical clinical signs of PD include motor symptoms such as bradykinesia, akinesia, and hypokinesia (slowness, absence and decreased amplitude of movement, respectively); resting tremor; freezing; and rigidity and postural instability, with a typically unilateral onset (Jankovic, 2008). Motor abnormalities can also be seen in reflexes, speech, swallowing, respiration, and eye movements (Jankovic, 2008). The particular clinical features as well as the severity and course of the disease can vary greatly between individuals due to poorly understood reasons (Obeso et al., 2017). The motor symptoms are typically also accompanied (and often preceded) by a wide variety of potentially disabling and distressing non-motor symptoms, such as a reduced sense of smell, sleep disorders, urinary and sexual dysfunction, depression, constipation, hallucinations, pain, anxiety, and cognitive dysfunction (Schapira et al., 2017).

PD is the second most common neurodegenerative disease (after Alzheimer's disease), affecting mainly the older population with incidence being low before the age of 50 years but increasing rapidly with age from there onwards (Ascherio and Schwarzschild, 2016). Recent estimates of the median lifetime risk are 2 % for men and 1.3 % for women aged 40 years in the United States (Ascherio and Schwarzschild, 2016). The estimated annual costs of PD reach over 10 billion dollars in the United States alone, and the disease has a significant negative impact on the quality of life of both patients and caregivers (Chen, 2010). Importantly, non-motor symptoms such as cognitive dysfunction and depression may have an even greater impact on worsening disability and quality of life than the classical motor symptoms (Hely et al., 2008; Chen, 2010). Known and suspected risk and protective factors for PD are diverse and numerous, and the interested reader is referred to a recent review by Ascherio and Schwarzschild (2016).

2.2.2. Pathophysiology of Parkinson's disease

Features, causes and mechanisms of dopaminergic neurodegeneration

As discovered by Oleh Hornykiewicz and others already in the 1960s (Hornykiewicz, 2006), the main hallmark of PD is the progressive death of nigrostriatal dopaminergic neurons with a still fundamentally unclear etiology. Another hallmark of PD diagnosis is the presence of intraneuronal protein inclusions termed Lewy bodies; despite not being specific to PD or strictly necessary for it, "synucleinopathies" of various forms are now

thought to be one of the most significant contributors to PD etiology (Michel et al., 2016; Obeso et al., 2017).

The loss of midbrain dopaminergic neurons in PD is markedly selective, with the greatest cell loss occurring in the SNC (Bernheimer et al., 1973; Fearnley and Lees, 1991). The average magnitude of neurodegeneration observed in *post mortem* patient studies has been approximately 50–90 % of SNC cells lost, depending on the SNC subregion (Fearnley and Lees, 1991; Hall et al., 2014), with more than 80 % of dopamine lost in the caudate nucleus and 99 % in the putamen (Bernheimer et al., 1973). Compared to normal aging, neurodegeneration in PD progresses exponentially and roughly ten times faster (Fearnley and Lees, 1991). Parkinsonian neurodegeneration also exhibits a different topography, affecting primarily the ventrolateral SNC, in contrast to the dorsolateral SNC being affected in normal aging (Fearnley and Lees, 1991). The mesolimbic pathway is also degenerated in PD but to a lesser degree (Hall et al., 2014). Remarkably, due to functional compensation motor symptoms appear only when cell loss in the SNC reaches approximately 50 %, accompanied by a 70–80 % loss of striatal dopamine (Bernheimer et al., 1973; Fearnley and Lees, 1991).

The current view of the etiology of PD holds that both genetic factors and environmental influences contribute to a common pathophysiological process (Obeso et al., 2017). Although most cases of PD are sporadic, numerous genetic causes underlying inherited PD and risk variants for sporadic PD have been identified (Martin et al., 2011; Ferreira and Massano, 2017). These include mutations in genes such as *SNCA* (encoding the protein α-synuclein, the main component of Lewy bodies), *LRRK2, VPS35, Parkin, DJ-1, PINK1,* and many others, often encoding proteins of poorly understood function. The influence of the environment is apparent from the numerous known environmental risk factors (Ascherio and Schwarzschild, 2016), but no specific toxin explaining any but a small fraction of cases has been identified (Obeso et al., 2017).

See Figure 3 for a summary of various known pathophysiological mechanisms underlying dopaminergic neurodegeneration in PD. These mechanisms include misfolding and enhanced aggregation of proteins, particularly α-synuclein, resulting in the disturbance of numerous cell processes and possibly spreading via secretory mechanisms (Lashuel et al., 2013; Michel et al., 2016); disruptions in protein degradation and recycling (autophagy-lysosome and proteasome systems; Michel et al., 2016); endoplasmic reticulum stress (Michel et al., 2016); mitochondrial dysfunction (Michel et al., 2016); oxidative stress, possibly caused by dysregulation of intracellular calcium signaling or glutamatergic excitotoxicity (Michel et al., 2016); and neuroinflammation (Hirsch and Hunot, 2009). Many of these phenomena are associated with proteins encoded by the various PD-associated genes (Ferreira and Massano, 2017) and, in particular, with pathological α-synuclein aggregation. Another fundamentally unclear question is the reason for the selective vulnerability of nigrostriatal dopaminergic neurons. Suggested explanations

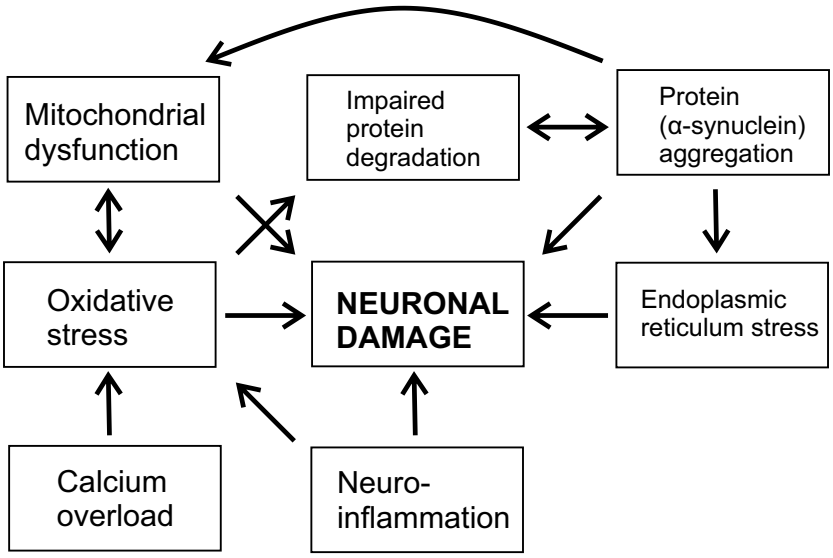

Figure 3. Simplified summary of some of the pathophysiological mechanisms of Parkinson's disease. Among key phenomena are pathological aggregation of proteins (in particular α-synuclein) and mitochondrial dysfunction, with numerous possible causes including gene mutations (not shown), impaired protein degradation due to dysfunctions in the proteasome and lysosome systems, and oxidative stress. Information sources: Hirsch and Hunot (2009), Martin et al. (2011), Michel et al. (2016)

include heavy energy demand and protein recycling burden due to highly branched axons, elevated basal mitochondrial stress due to calcium-dependent pacemaking, and numerous others (Brichta and Greengard, 2014; Obeso et al., 2017).

Consequences of dopaminergic neurodegeneration

The classical view of parkinsonian basal ganglia dysfunction is that the loss of striatal dopaminergic modulation results in decreased direct pathway activity and increased indirect pathway activity, both leading to excessive activity of the inhibitory basal ganglia output nuclei (SNR and GPi) and thus to excessive inhibition of their target motor regions (Figure 4; Albin et al., 1989; DeLong, 1990). Notably, recent rodent evidence suggests that decreased firing of dMSNs may be the predominant mechanism (Ryan et al., 2018). Alterations in synaptic plasticity are also thought to be involved. In the rodent striatum, corticostriatal synapses of both dMSNs and iMSNs exhibit bidirectional synaptic plasticity – reversible long-term potentiation (LTP) and long-term depression (LTD) – that is controlled by dopamine and other neurotransmitters (Shen et al 2008, 2015). After dopamine depletion, both LTP and LTD are lost in MSNs (Calabresi et al., 1992; Centonze et al., 1999; Picconi et al., 2003; Picconi et al., 2011). Further studies, separately investigating the two MSN classes, suggest that the loss of dopamine leads to an imbalance where dMSNs exhibit only LTD, while iMSNs exhibit only LTP (Shen et al., 2008; Belujon et al., 2010; Thiele et al., 2014).

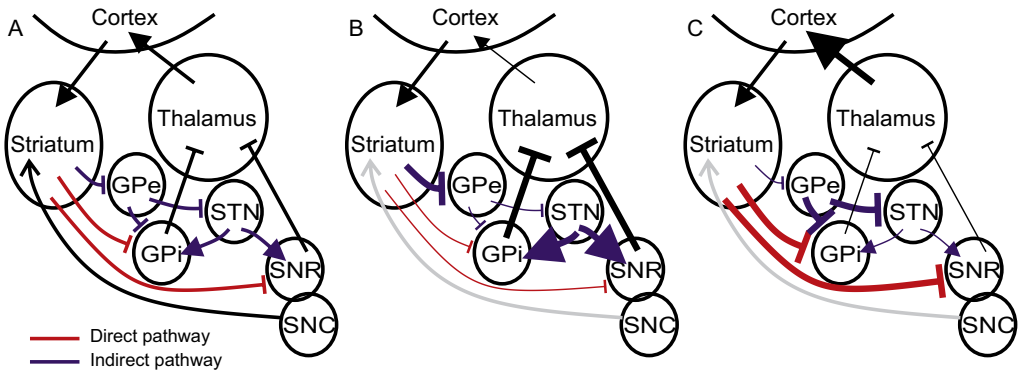

Figure 4. Classical model of basal ganglia dysfunction in Parkinson's disease and hyperkinetic disorders as proposed by e.g., DeLong (1990). **A**: In normal conditions, dopamine released from nigrostriatal projections activates the striatal output neurons of the direct pathway and inhibits the output neurons of the indirect pathway. A balance is maintained between the opposite effects of the two pathways on the basal ganglia output nuclei (GPi, SNR) and on basal ganglia output. **B**: In parkinsonism, the loss of nigrostriatal dopamine release results in reduced activity of direct pathway neurons and increased activity of indirect pathway neurons. Both alterations lead to overactivation of the output nuclei and thus to overinhibition of the target regions. **C**: In hyperkinetic disorders, such as levodopa-induced dyskinesia, uncontrolled striatal dopaminergic stimulation overactivates direct pathway neurons and overinhibits indirect pathway neurons. Both alterations lead to overinhibition of the output nuclei and to abnormal disinhibition of the target regions. Schematic representation of selected basal ganglia projections shown. Effects of abnormal basal ganglia output on information processing in cortical areas are not considered. GPe = external segment of the globus pallidus; GPi = internal segment of the globus pallidus; STN = subthalamic nucleus; SNR = substantia nigra pars reticulata; SNC = substantia nigra pars compacta

These latter findings appear remarkably in line with the classical model, although note that one study observed a similar loss of LTP in both MSN subtypes (Shen et al., 2015).

The current view of basal ganglia connectivity has, however, begun to move away from the concept of strict pathway segregation (Cazorla et al., 2014). Reflecting this, changes in discharge patterns, rather than simply in neuronal activity rates, have been suggested to underlie parkinsonism. More specifically, current evidence suggests that dopaminergic denervation results in increased firing of MSNs, increased coupling between cortical and basal ganglia activity, and widespread excessive synchronization and oscillatory activity throughout the basal ganglia, where neuronal activity normally is generally asynchronous (Hammond et al., 2007; Ko et al., 2014; Cagnan et al., 2015; Neumann et al., 2016). In particular, exaggerated synchronized oscillations at the beta frequency band (8–30 Hz), generated locally but propagating through the basal ganglia-cortical loop, are correlated with parkinsonian motor impairment in both animal models and untreated patients

(Hammond et al., 2007; Neumann et al., 2016). The role of dopamine in motor control may thus be to dampen excessive synchronization within the basal ganglia and to enable dynamic modulation of connectivity (Hammond et al., 2007; Cagnan et al., 2015). The loss of tonic dopamine release could then result in the striatum becoming unable to filter and integrate cortical motor command signals, leading to a lack of coordinated activity between direct and indirect pathways and an inability to select, initiate, and terminate motor action sequences (Hammond et al., 2007; Calabresi et al., 2014; Ko et al., 2014; Cagnan et al., 2015).

Non-motor and non-dopaminergic pathophysiology

Reflecting the wide variety of possible non-motor symptoms of PD (Schapira et al., 2017), numerous neurodegenerative and other disturbances besides those related to motor control have been described, both within and outside of the basal ganglia. Dopaminergic neurodegeneration, including not only nigrostriatal degeneration but also damage and/or imbalances in mesolimbic, mesocortical, and other dopaminergic systems, may underlie such varied non-motor symptoms as mood and anxiety disorders, cognitive disorders, sleep disorders, apathy, fatigue, visual disturbances, and pain (Barone, 2010; Schapira et al., 2017). Degeneration of serotonergic neurons in the raphe nuclei and their projections to the cortex, the basal ganglia, and other brain areas has been observed in PD patients, and may be linked to affective disorders, pain, sleep disorders, and fatigue (Barone, 2010; Pagano et al., 2017; Schapira et al., 2017). Noradrenergic neurons in the locus coeruleus also degenerate in PD patients, possibly contributing to non-motor symptoms (Barone, 2010; Schapira et al., 2017). Excessively enhanced glutamatergic activity may contribute to cognitive impairment and depression (Barone, 2010), while GABAergic dysfunction affecting both MSNs and interneurons is thought to contribute to aberrant synchronization as well as non-motor symptoms (Barone, 2010; Gittis and Kreitzer, 2012).

The role of the cholinergic system in PD pathophysiology merits particular mention. A classical hypothesis has maintained that a striatal hypercholinergic tone and a resulting disturbed striatal dopamine-acetylcholine balance contributes significantly to parkinsonian motor symptoms (Barbeau, 1962). While the role of acetylcholine in the striatum has subsequently been much revised (Oldenburg and Ding, 2011; see also 2.4.2.), inhibition of cholinergic interneuron activity can alleviate motor dysfunction in parkinsonian animal models (Maurice et al., 2015), suggesting that hyperactive cholinergic neurotransmission could indeed contribute to parkinsonism. In addition, a loss of cortical cholinergic innervation in PD patients has been long recognized, and the resulting depletion of cortical acetylcholine has been suggested to contribute to the cognitive impairment commonly associated with PD (Barone, 2010; Schapira et al., 2017). Indeed, drugs enhancing cholinergic function have long been used for treating PD-associated dementia. Moreover, cholinergic dysfunction has been suggested to contribute to various irregularities such as gastrointestinal problems, sleep disturbances, and olfactory dysfunction (Barone, 2010; Schapira et al., 2017). Finally, drugs affecting nicotinic acetylcholine receptors

can be neuroprotective (see 2.4.4.), suggesting a significant role for nAChR-mediated neurotransmission in PD pathophysiology.

2.2.3. Current treatment of Parkinson's disease and future perspectives

The remarkable symptomatic efficacy achieved in PD with the dopamine precursor molecule levodopa (L-dopa, L-3,4-dihydroxyphenylalanine) was discovered already in the 1960s, not long after the critical role of dopamine loss became clear (Hornykiewicz, 2006). Today, levodopa remains the most effective and best-tolerated treatment against the motor symptoms of PD (Fox et al., 2018). Levodopa is administered orally, usually along with benserazide or carbidopa, inhibitors of aromatic-L-amino-acid decarboxylase (AADC), to block peripheral metabolism (Lewitt, 2015). In the brain AADC converts levodopa to dopamine, with the therapeutic effect stemming in particular from dopamine synthesis in the striatum, performed within the remaining dopamine terminals as well as serotonin terminals (Lewitt, 2015; see also 2.3.2.). The levodopa-derived dopamine acts to replace lost endogenous dopamine, relieving motor symptoms at least in part through the suppression of excessive basal ganglia-cortex synchrony (Hammond et al., 2007). Treatment with levodopa is usually markedly effective, particularly in the beginning of the treatment, and was at first even hailed as a "miracle" (Hornykiewicz, 2006). However, as soon became apparent, long-term treatment with levodopa is often associated with a gradual appearance of significant motor and non-motor complications (Aquino and Fox, 2015).

Levodopa-induced motor complications include levodopa-induced dyskinesia (discussed in detail in the following section) as well as motor fluctuations, referring to variable or unpredictable therapeutic effects (Aquino and Fox, 2015). Motor fluctuations can include delayed or even completely failed symptomatic relief; unpredictable or sudden "wearing off", referring to the re-emergence of parkinsonian motor symptoms; and potentially unpredictable "on-off" fluctuations between mobile and immobile states (Aquino and Fox, 2015). Severe on-off fluctuations have fortunately become rare due to lower levodopa dosing strategies (Aquino and Fox, 2015). Long-term levodopa treatment often also results in the induction and fluctuation of non-motor symptoms, with neuropsychiatric, autonomic, and sensory symptoms either worsening during periods of wearing off or, conversely, appearing or worsening during periods of motor improvement (Bastide et al., 2015; Aquino and Fox, 2015).

To avoid complications, a traditional approach in the treatment of Parkinson's disease has been to delay levodopa treatment as long as possible. However, more recently it has been suggested that this represents an unfounded "levodopa phobia" and that early treatment with levodopa is more beneficial (Vlaar et al., 2011; Giannakis et al., 2018; Matarazzo et al., 2018). More specifically, it has been argued that worsening levodopa-induced complications are more related to disease progression than the treatment itself; that delaying levodopa treatment does in fact not postpone motor fluctuations; that levodopa is not toxic to

dopaminergic neurons contrary to earlier beliefs; and that while LID is common, it is often not disabling and should be tolerated as long as effective motor symptom alleviation is achieved (Vlaar et al., 2011; Matarazzo et al., 2018). Levodopa-induced complications can also often be mitigated by using a lower dose, but typically with the drawback of reduced symptomatic efficacy (Manson et al., 2012; Matarazzo et al., 2018).

In addition to levodopa, a number of other pharmacological treatments exist for motor symptoms of Parkinson's disease. Dopamine receptor agonists, catechol-O-methyltransferase inhibitors and monoamine oxidase (MAO) B inhibitors are often used and effective; other options include anticholinergic drugs and amantadine. Different drugs are often used in combination, particularly in later stages of treatment; for detailed up-to-date treatment guidelines see Fox et al. (2018). In addition, a wide range of pharmacological treatments can be used to mitigate various non-motor symptoms of the disease (Schapira et al., 2017). Finally, deep brain stimulation (DBS) of the GP or the STN has been established as a remarkably effective treatment for parkinsonian motor symptoms, with the effects likely mediated by modulation of pathological synchronized oscillations (Fasano et al., 2012; Guridi and Alegre, 2017).

Despite the variety of options for treating the symptoms of PD, none of them affect the progression of the disease. While dopaminergic medication can often provide adequate control of motor symptoms, the treatment of worsening non-motor symptoms such as cognitive dysfunction is challenging. Great efforts have been expended – thus far unsuccessfully – in the search of therapies that would significantly slow or stop the degeneration of dopaminergic neurons and the worsening clinical status of the patient. A plethora of treatments with a wide variety of mechanisms of action have been found promising in preclinical studies but clinically ineffective (AlDakheel et al., 2014; Fox et al., 2018). The disappointing clinical results may stem in part from issues such as the somewhat questionable translational validity of neurotoxin-based animal models of PD or the challenges of measuring neuroprotection in living patients (AlDakheel et al., 2014). Further clinical studies are continuing, and some novel targets being investigated include neurotrophic factors, inflammatory pathways, calcium channels, and α-synuclein aggregation (AlDakheel et al., 2014) as well as nicotinic acetylcholine receptors (see 2.4.4.).

2.3. Levodopa-induced dyskinesia

2.3.1. Clinical features and epidemiology

Levodopa-induced dyskinesia refers to abnormal involuntary movements that typically arise acutely in response to a dose of levodopa. LID can be further classified based on time of appearance (Aquino and Fox, 2015; Bastide et al., 2015). Peak-dose dyskinesia, appearing at the peak of levodopa action, is the most common form and typically manifests as chorea (involuntary, irregular, abrupt, and rapid movements often flowing from one body part

to another), dystonia (sustained muscle contractions) or, in severe cases, ballism (large limb movements). Other possible forms of peak-dose dyskinesia include involuntary eye movements, irregular breathing, and involuntary muscle twitching. Peak-dose dyskinesia is the most studied subcategory of LID and also the subject of the present studies. Other types of LID include diphasic dyskinesia, appearing just before and at the end of the therapeutic effect, and off-period dystonia that appears outside of the therapeutic effect.

The incidence of LID increases with continuing levodopa treatment, and risk factors include young age, female sex, and disease duration and severity (Ahlskog and Muenter, 2001; Manson et al., 2012; Matarazzo et al., 2018). The dose and duration of levodopa treatment are typically considered particularly significant risk factors (Manson et al., 2012; Matarazzo et al., 2018), although interestingly a recent meta-analysis found no evidence of a correlation between LID and treatment dose or duration (Giannakis et al., 2018). Genetic risk factors include those associated with genetic parkinsonism as well as polymorphisms in genes encoding dopamine receptors, opioid receptors, and the brain-derived neurotrophic factor (BDNF; Bastide et al., 2015). LID has traditionally been considered a significant complication, and levodopa treatment has thus been delayed for as long as possible (Ahlskog and Muenter, 2001; but see 2.2.3. above). However, some more recent studies have brought into question the traditional views on the clinical significance of LID. Dyskinesia was more common during the early levodopa era than in modern use, possibly due to a more severe average disease progression at treatment initiation (Ahlskog and Muenter, 2001). Also in modern treatment LID of some severity seems to affect the clear majority of patients after long enough use, with such reported rates of dyskinesia as every patient after 20 years (Hely et al., 2008), about 90 % of patients after 9–15+ years of levodopa treatment (Ahlskog and Muenter, 2001), about 80 % of patients after 5–10 years (Chapuis et al., 2005), and about 80 % of patients after 10 years (Hauser et al., 2007). However, also somewhat lower incidences, such as 60 % of patients after 10 years (Van Gerpen et al., 2006) or only 35 % of patients after 10 years and 60 % after 15 years (Sato et al., 2006), have been reported in some cohorts.

A more controversial question seems to be the incidence of clinically significant dyskinesia. Most earlier studies did not specifically investigate the clinical significance of LID (Ahlskog and Muenter, 2001), and some modern studies have reported low incidences of significant LID. For example, Van Gerpen et al. (2006) reported significant dyskinesia that could not be adequately controlled by medication adjustments only in 12 % of patients after 10 treatment years (about 60 % with any LID); Hauser et al. (2007) reported, albeit in a small cohort, that only about 25 % of patients showed at least moderately disabling LID after 10 treatment years (about 80 % with any LID); and a number of studies have reported no significant impact by LID on different quality of life measures after various treatment lengths (Marras et al., 2004; Rahman et al., 2008; Winter et al., 2010). On the other hand, a number of studies have also reported significant detrimental impacts by LID on quality of life measures (Chapuis et al., 2005; Péchevis et al., 2005; Reuther et al., 2007). Recently,

a retrospective study undertaken in a large cohort of PD patients aimed at systematically investigating the impact of different motor fluctuations on health-related quality of life (Perez-Lloret et al., 2017). In this study, nearly 70 % of patients experienced some degree of LID after 9+ treatment years, and both LID and other motor fluctuations were found

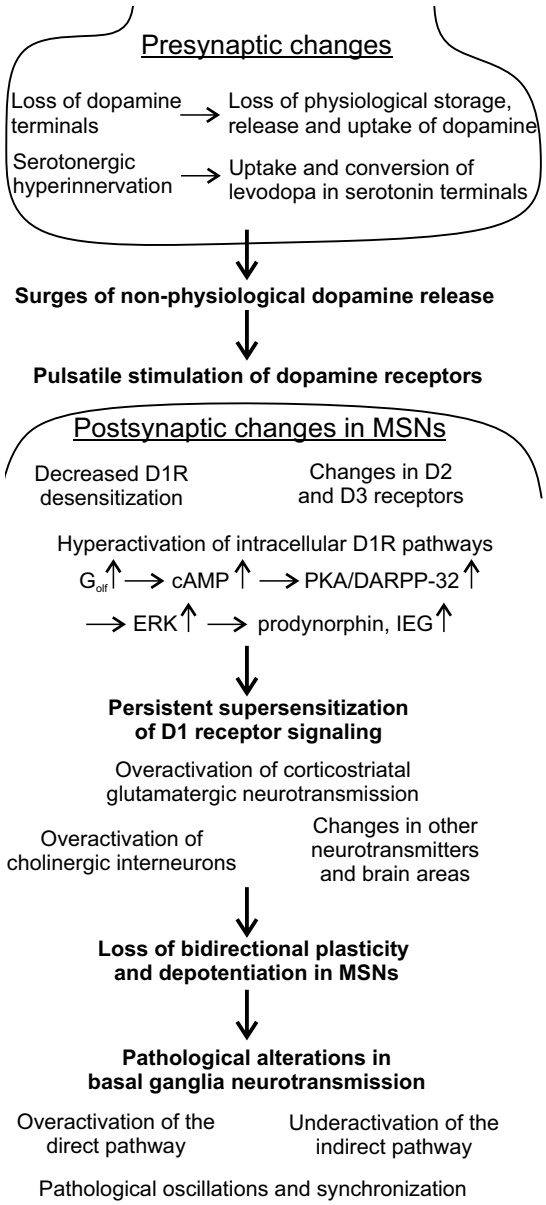

Figure 5. Pathophysiological mechanisms of levodopa-induced dyskinesia. Abnormal presynaptic handling of levodopa and hyperactivation of D1 receptor signaling leads to dysfunctional synaptic plasticity and abnormal basal ganglia neurotransmission. BDNF = brain-derived neurotrophic factor; cAMP = cyclic adenosine monophosphate; D1R = dopamine D1 receptor; ERK = extracellular signal-regulated kinase; IEG = immediate early gene; PKA = protein kinase A

to significantly and independently correlate with quality of life. In addition, it was found that the severity of LID (as opposed to LID duration) may be the most important factor determining the subjective impact of LID. Thus, it appears that a perhaps smaller than previously thought but still significant proportion of PD patients suffer from levodopa-induced dyskinesia severe enough to detrimentally affect quality of life.

2.3.2. Pathophysiology of peak-dose LID

The pathophysiology of levodopa-induced dyskinesia has been extensively studied; for a comprehensive and relatively recent review, see Bastide et al. (2015). The role of pre- and postsynaptic changes in LID has been the subject of great debate during the last few decades, but an explosion of evidence during the last ten years or so indicates – perhaps in the end unsurprisingly – critical contributions by both. On the most general level, it can be stated that LID is thought to develop in response to non-physiological striatal release of dopamine and pulsatile stimulation of dopamine receptors that result from the repeated administration of exogenous levodopa. Postsynaptic changes in striatal MSNs, in particular supersensitization of D1R-mediated signaling, combined with abnormalities in various other neurons and neurotransmitter systems, then result in alterations in synaptic plasticity and the neuronal firing patterns associated with basal ganglia neurotransmission. The current view of LID pathophysiology is summarized in Figure 5, and the underlying evidence will next be reviewed in some detail.

Abnormal presynaptic handling of levodopa

The effects of levodopa *in vivo* result from its conversion to dopamine in the brain, as demonstrated already in many early findings of increased striatal dopamine release after levodopa administration (see references in Abercrombie et al., 1990) and inhibition of its behavioral effects when the conversion is blocked (Ungerstedt, 1971). More recently, many *in vivo* microdialysis studies have shown increased striatal extracellular dopamine levels after both local and systemic administration of levodopa in intact rats (Abercrombie et al., 1990; Sarre et al., 1997; Opacka-Juffry et al., 1998; Shui et al., 2000) as well as in rats with unilateral 6-hydroxydopamine (6-OHDA) lesions (Abercrombie et al., 1990; Brannan et al., 1990; Brannan et al., 1998; Meissner et al., 2006; Buck and Ferger, 2008; Lindgren et al., 2010). On the other hand, dyskinesia expressed after levodopa administration is associated with increased plasma and brain levodopa levels in human patients (Olanow et al., 1991), monkeys lesioned with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Huot et al., 2012; Porras et al., 2014), and 6-OHDA-lesioned hemiparkinsonian rats (Carta et al., 2006). Moreover, in 6-OHDA-lesioned rats LID is associated with increased striatal dopamine release (Brannan et al., 1998; Meissner et al., 2006; Buck and Ferger, 2008; Lindgren et al., 2010), and blocking the conversion of levodopa to dopamine prevents LID (Buck and Ferger, 2008). Similarly, levodopa-induced putaminal dopamine release, measured with positron emission tomography (PET) imaging, correlates with LID severity in PD patients (Pavese et al., 2006; Politis et al., 2014). These findings strongly suggest that the primary

cause of LID is indeed the striatal release of levodopa-derived dopamine. Note, however, a contrasting finding in MPTP-lesioned monkeys, where levodopa administration produced LID but no increased striatal dopamine release (Porras et al., 2014). Some intriguing findings also suggest increased central levodopa bioavailability as a possible mechanism for LID susceptibility (Carta et al., 2006; Lindgren et al., 2010).

The development of LID appears to require conditions of dopaminergic denervation, as dyskinesia is not induced by levodopa administration in non-parkinsonian humans (Arts et al., 1991), monkeys (except in some rare reports; Togasaki et al., 2001), or rodents (Winkler et al., 2002; Lundblad et al., 2004; Buck and Ferger, 2008; Francardo et al., 2011). The degree of denervation also affects LID severity. Monkeys and rodents suffering from near-total dopaminergic denervation are typically more prone to develop severe LID than animals suffering from a partial lesion (Schneider, 1989; Winkler et al., 2002; Lundblad et al., 2004; Francardo et al., 2011; however, see Guigoni, Dovero, et al., 2005 for a contrasting finding in monkeys). PET studies suggest that the situation is similar in PD patients. Dopamine transporter (DAT) activity in the putamen is negatively correlated with LID severity (Linazasoro et al., 2004) and lower in dyskinetic than non-dyskinetic patients (Hong et al., 2014), suggesting a correlation between dopamine terminal loss and LID.

Importantly, significant changes in the presynaptic handling of levodopa besides the simple loss of dopamine terminals appear to underlie LID. In most microdialysis studies in 6-OHDA-lesioned rats the levodopa-induced striatal dopamine release was in fact much larger in the denervated hemisphere (Abercrombie et al., 1990; Meissner et al., 2006; Buck and Ferger, 2008; Lindgren et al., 2010). Similarly, human PET imaging findings suggest that levodopa-induced striatal dopamine release increases as PD progresses (de la Fuente-Fernández et al., 2004; Pavese et al., 2006). Furthermore, dyskinetic patients show larger, but possibly shorter-lived, increases in striatal levels of levodopa-derived dopamine than non-dyskinetic patients (de la Fuente-Fernández et al., 2004; Politis et al., 2014). The critical role of the state of the presynaptic dopamine terminals was convincingly demonstrated in a study where no LID was observed in rats after a functional knockdown of dopamine synthesis which left the dopamine terminals themselves intact (Ulusoy et al., 2010). Notably, LID was absent even in rats previously primed for dyskinesia with a dopamine agonist, suggesting that if the presynaptic dopamine terminals remain intact, levodopa-derived dopamine can be properly stored and released and LID avoided even if postsynaptic dyskinesiogenic changes have occurred.

In summary, current evidence suggests that the progressive dopamine terminal loss in PD results in increasing abnormalities in the presynaptic handling (transport, storage, and release) of levodopa and levodopa-derived dopamine, leading to larger and possibly shorter-lived transient increases in striatal dopamine release. The resulting pulsatile stimulation of postsynaptic MSN dopamine receptors is then suggested to lead to increasingly more severe LID (Stoessl, 2015). What, then, are the mechanisms of abnormal dopamine release

that become progressively more dominant as nigrostriatal dopamine terminals are lost? Although reduced dopamine transporter function likely plays a role (Troiano et al., 2009), extensive evidence points to a major contribution by striatal 5-hydroxytryptamine (5-HT, serotonin) terminals.

The possibility of levodopa being converted to dopamine in non-physiological sites was suggested already during the earliest studies, given its effects even when dopamine terminals are completely lost (see Duvoisin and Mytilineou, 1978 and references therein). Serotonin terminals were considered one possibility, since AADC (responsible also for 5-HT synthesis) was known to be expressed in serotonergic neurons, and indirect evidence existed that levodopa might be handled by them (see references in Tison et al., 1991). Later, direct immunohistochemical evidence for the presence of levodopa-derived dopamine in serotonergic neurons of the raphe nuclei and in striatal serotonin terminals was obtained (Tison et al., 1991; Arai et al., 1994; Arai et al., 1995). Further confirmation was obtained in microdialysis studies of 6-OHDA-lesioned rats, where striatal levodopa-induced dopamine release was significantly reduced by lesioning of the serotonergic system (Tanaka et al., 1999) and by stimulation of inhibitory 5-HT1A autoreceptors (Kannari et al., 2001). More recent rodent evidence suggests that levodopa-induced dopamine release may partially derive from serotonin terminals also in the intact striatum (Nevalainen et al., 2014).

Levodopa-derived dopamine thus appears to be able to act as a "false transmitter", synthesized in and released from serotonin terminals. This could certainly have beneficial therapeutic consequences, particularly in late-stage PD where few dopamine terminals remain. However, many studies suggest that this non-physiological release of dopamine is also critical for LID. Foremost, the induction and expression of LID in 6-OHDA-lesioned rats is almost entirely blocked by both non-specific lesioning of the serotonergic system (Carta et al., 2007) and specific lesioning of raphestriatal serotonergic projections (Eskow et al., 2009). Further evidence includes findings in many studies that agonists of the inhibitory 5-HT1A and 5-HT1B autoreceptors can attenuate LID and associated striatal dopamine release in 6-OHDA-lesioned rats and MPTP-lesioned monkeys, whether administered systemically (e.g., Bibbiani et al., 2001; Carta et al., 2007; Munoz et al., 2008; Lindgren et al., 2010) or directly to the raphe nuclei or the striatum (Eskow et al., 2009; Bishop et al., 2009). Note, however, that in some studies this was associated with a decreased therapeutic effect, suggesting that serotonin terminals indeed participate also in the beneficial pro-motor effects of levodopa (e.g., Iravani et al., 2006; Bezard et al., 2013). Finally, PET imaging studies have shown that serotonin terminals in the striatum and the GP and their function are relatively more preserved in dyskinetic than non-dyskinetic PD patients, with 5-HT1A agonist treatment reducing levodopa-induced dopamine release (Politis et al., 2014; Smith et al., 2015).

Considerable evidence thus suggests the release of levodopa-derived dopamine from raphestriatal serotonin terminals as an essential mechanism underlying LID. This non-

physiological dopamine release lacks the D2 autoreceptor-mediated feedback control exhibited by dopamine terminals and may thus result in large, uncontrolled increases in extracellular dopamine (Carta et al., 2007). Note also that besides the striatum, abnormal levodopa-induced dopamine release from serotonin terminals occurs also at least in the SN, hippocampus, and prefrontal cortex (Navailles et al., 2013). Various 5-HT1 receptor agonists, in turn, may suppress this non-physiological dopamine release and LID *via* the activation of various presynaptic and somatodendritic autoreceptors and heteroreceptors (Iderberg et al., 2015). Changes in the expression and function of the DAT, the serotonin transporter (SERT) and the noradrenaline transporter may also contribute to LID (Conti et al., 2016).

Although PET imaging studies suggest global serotonin terminal degeneration in PD (Pagano et al., 2017), local serotonergic hyperinnervation may occur. In rodents, striatal serotonergic hyperinnervation has been observed after both dopaminergic denervation alone (Maeda et al., 2003; Bez et al., 2016) and subsequent levodopa treatment (Maeda et al., 2005; Rylander et al., 2010). Similarly, increased serotonergic innervation was observed in monkeys after MPTP lesioning and was further increased by levodopa treatment (Zeng et al., 2010). Furthermore, increased SERT binding in *post mortem* brain tissue was observed in dyskinetic rats (striatum and cortex), MPTP-lesioned monkeys (striatum and GP), and dyskinetic PD patients (striatum and GP; Rylander et al., 2010). Serotonin terminal sprouting in the striatum could thus constitute a mechanism for the increased non-physiological release of dopamine as LID develops. Interestingly, the levodopa-induced SERT upregulation in rats was paralleled by upregulation of *Bdnf* mRNA, suggesting BDNF-mediated maladaptive plasticity as a mechanism for the serotonergic hyperinnervation (Rylander et al., 2010). In line with this, a recent study found that BDNF overexpression in rats increased striatal serotonergic fiber sprouting and susceptibility to LID (Tronci et al., 2017). Somewhat in contrast, however, carriers of a *Bdnf* gene polymorphism associated with lower BDNF secretion have a higher risk for developing LID (Foltynie et al., 2009; Kusters et al., 2018).

Changes in dopaminergic signaling in striatal medium spiny neurons

Persistently sensitized D1R-mediated signaling in dMSNs is usually considered the most critical postsynaptic phenomenon underlying LID, regardless of whether it is thought to result primarily from the levodopa-induced pulsatile stimulation (Stoessl, 2015) or already from the loss of striatal dopaminergic innervation (Nadjar et al., 2009). While striatal D1R numbers are mostly unchanged in PD patients (Hurley et al., 2001), studies in animal models suggest that their signaling is enhanced (Aubert et al., 2005). This is at least in part the result of decreased D1R desensitization due to increased membrane expression and reduced internalization (Guigoni et al., 2007; Berthet et al., 2009). In addition, the expression patterns of a number of desensitization-regulating kinases and arrestins are changed by dopaminergic lesions and subsequent levodopa treatment (Bezard et al., 2005; Ahmed et al., 2008; Ahmed et al., 2015). Dysregulated lateral diffusion along the

cell membrane (Porras et al., 2012) and inhibited proteasomal degradation (Berthet et al., 2012) have also been indicated as mechanisms of D1R sensitization. Finally, the G-protein mediator of D1R activation $G\alpha_{olf}$ is upregulated in the striatum of PD patients and animal models, possibly contributing to the supersensitized signaling (Hervé et al., 1993; Corvol et al., 2004; Alcacer et al., 2012).

The intracellular consequences of the supersensitized D1R signaling that are closely associated with LID include increased expression of prodynorphin (Cenci et al., 1998) and a number of immediate-early gene (IEG) products such as FosB/ΔFosB (Andersson et al., 1999), Zif-268 (Carta et al., 2005), Arc (Sgambato-Faure et al., 2005), and c-Fos (Santini et al., 2007). The critical role of ΔFosB overexpression in particular is reflected in striatal ΔFosB overexpression alone resulting in LID-like behavioral sensitization to levodopa (Cao et al., 2010). Recent evidence further suggests that ΔFosB accumulation in MSNs results in altered electrical properties leading to LID (Engeln et al., 2016). In addition, comprehensive gene expression studies in rodents have found levodopa treatment and LID to be associated with changes in the expression of hundreds of genes, implicating among other things increased transcriptional and synaptic activity (Konradi et al., 2004; El Atifi-Borel et al., 2009; Heiman et al., 2014).

Rodent studies have elucidated in detail the intracellular pathways, downstream of the supersensitized D1 receptors, that are associated with LID. In brief, sequential as well as parallel hyperactivation of $G\alpha_{olf}$, cAMP production, PKA, DARPP-32, and the extracellular signal-regulated protein kinase (ERK) occurs in response to dyskinesia-inducing levodopa administration (Gerfen et al., 2002; Picconi et al., 2003; Pavón et al., 2006; Santini et al., 2007; Westin et al., 2007; Santini, Alcacer, et al., 2009; Alcacer et al., 2012). This activation cascade results in altered transcription of prodynorphin and the IEGs, at least in part through dysregulation of the mitogen- and stress-activated kinase 1 and altered histone H3 phosphorylation (Santini et al., 2007; Darmopil et al., 2009; Feyder et al., 2016). Other key components modulating the LID-associated D1R-mediated ERK pathway activation include the Ras-guanine nucleotide-releasing factor 1 (Fasano et al., 2010) and mGluR5 glutamate receptors (Fieblinger et al., 2014). ERK-induced activation of the mammalian target of rapamycin complex 1 and resulting altered translation also plays a role (Santini, Heiman, et al., 2009). These extensive rodent findings have been in part replicated in primates: elevated striatal ΔFosB levels are found in PD patients (Tekumalla et al., 2001), and LID in MPTP-lesioned monkeys has been associated with increased prodynorphin and ΔFosB expression (Berton et al., 2009) as well as increased cAMP/PKA/DARPP-32 and ERK signaling (Santini et al., 2010). One final functional result of these alterations may be aberrant synaptic plasticity (see 2.3.2.). Finally, ERK hyperactivity appears to not persist during long-term levodopa treatment, suggesting that the ERK pathway may be primarily involved in sensitization while DARPP-32-mediated signaling may be responsible for LID maintenance (Santini et al., 2010; Ahmed et al., 2015).

Notably, many of the aforementioned cellular alterations as well as related gene expression changes have been found to occur and/or persist specifically in D1R-expressing dMSNs (Carta et al., 2005; Sgambato-Faure et al., 2005; Darmopil et al., 2009; Santini, Alcacer, et al., 2009; Santini et al., 2012; Heiman et al., 2014). Recent optogenetic experiments further suggest that although other mechanisms also contribute, LID is primarily associated with the activation of a subset of D1R-expressing MSNs in the dorsal striatum, selective re-activation of which is sufficient to induce dyskinesia in parkinsonian rodents (Hernández et al., 2017; Perez et al., 2017; Girasole et al., 2018; Ryan et al., 2018). LID is also associated with increased GABA release in the SNR, possibly reflecting the dMSN hyperactivation (Bido et al., 2011). The roles of other dopamine receptors in LID pathophysiology have been much less studied. Although the expression of D2 receptors is not changed by levodopa treatment (Aubert et al., 2005; Guigoni et al., 2007), they are thought to contribute to LID given such findings as D2R agonist-induced dyskinesia in animal models (Blanchet et al., 1993), inhibition of LID by selective iMSN stimulation (Alcacer et al., 2017), and association of LID with changes in D2R-specific signaling proteins (Gold et al., 2007). D3 receptors (D3R) also likely have a role, as LID is associated with increased striatal D3R expression in monkeys (Bézard et al., 2003), and selective D3R ligands modulate LID in animal models (Bézard et al., 2003; Kumar et al., 2009). Interestingly, D3R-D1R interactions may contribute to D1R sensitization and thus LID *via* regulation of membrane anchoring and internalization (Berthet et al., 2009). Furthermore, levodopa administration can increase corticostriatal BDNF release and trigger BDNF-mediated D3R overexpression (Guillin et al., 2001); BDNF might thus have a role in D1R sensitization. Finally, D4 receptors may also contribute to LID (Huot et al., 2015).

Changes in other neurotransmitter systems and brain regions

Acetylcholine released by striatal cholinergic interneurons exerts a complex modulation over corticostriatal neurotransmission (see 2.1.3.), and ChIs play a significant role also in LID. In rodents, striatal dopamine depletion results in increased acetylcholine release (Ding et al., 2006). Subsequent dyskinesia-inducing levodopa treatment was found to result in a switch in ERK hyperactivation from MSNs to ChIs, leading to enhanced ChI firing both basally and in response to dopamine (Ding et al., 2011). Very interestingly, selective elimination of striatal ChIs before levodopa treatment almost completely blocked the development LID without affecting therapeutic efficacy (Won et al., 2014). A recent optogenetic study by Bordia et al. (2016) further characterized the role of ChIs in LID. Short pulse stimulation of ChIs, presumably generating a limited amount of acetylcholine release, increased LID *via* mAChRs and even induced dyskinesia in previously non-dyskinetic animals. In contrast, longer pulse stimulation, presumably resulting in greater acetylcholine release and possibly desensitization of nicotinic receptors, reduced LIDs via both mAChRs and nAChRs. In rodents, at least, acetylcholine released by ChIs can thus affect LID both through mAChRs, expressed directly on MSNs as well as on multiple other striatal neuron and terminal types (Oldenburg and Ding, 2011), and through nAChRs. See 2.4.4. for a detailed discussion on nAChRs and LID.

Overactivation of glutamatergic neurotransmission in the basal ganglia also contributes significantly to LID (Robelet et al., 2004). While dopamine depletion in rodents results in a dramatic pruning of corticostriatal synapses, dyskinesiogenic levodopa administration induces enlargement of the remaining spines and facilitation of synaptic transmission in dMSNs while restoring spines but inhibiting synaptic transmission in iMSNs (Suarez et al., 2016). Numerous changes in glutamate receptor composition, localization, and activity also occur in association with dopaminergic denervation and LID (Mellone and Gardoni, 2018). Other neurotransmitter systems thought to contribute to LID expression include the serotonergic system (Navailles et al., 2013; see also 2.3.2.) as well as the opioid, endocannabinoid, and noradrenergic systems (Fox and Brotchie, 2014). Interestingly, also histaminergic neurons that densely innervate the striatum are able to take up levodopa and release levodopa-derived dopamine, suggesting that they could contribute to non-physiological dopamine release (Yanovsky et al., 2011). Besides the striatum, overactivation of other basal ganglia structures may underlie various levodopa-induced non-motor complications (Guigoni, Li, et al., 2005). Also many brain areas outside of the basal ganglia are involved in LID, with widespread levodopa-induced changes observed in IEG expression (Bastide et al., 2014) and monoamine release (Navailles et al., 2013; Engeln et al., 2015). Recent studies also indicate the involvement of various cortical areas (see next section). Finally, non-neuronal phenomena that may contribute to LID include alterations in cerebral blood flow, angiogenesis, and blood-brain barrier permeability (Ohlin et al., 2012).

Alterations in basal ganglia neurotransmission

The classical model of hyperkinetic disorders such as LID postulates that excessive dopaminergic stimulation of the D1R-expressing dMSNs and, in parallel, excessive inhibition of the D2R-expressing iMSNs both lead to overinhibition of the basal ganglia output nuclei (GPi/SNR) and thus to pathological disinhibition of the target motor regions (Figure 4; DeLong, 1990). Supporting the model are findings of decreased firing frequency and altered firing patterns of single GPi neurons during dopamine receptor-mediated dyskinesia in parkinsonian monkeys and in PD patients (Papa et al., 1999; Boraud et al., 2001; Lee et al., 2007). Also in line is the extensive evidence demonstrating supersensitization of D1R-mediated signaling in dMSNs (see 2.3.2.) as well as recent optogenetic findings showing that levodopa increases dMSN firing while decreasing iMSN firing in rodents (Ryan et al., 2018).

Aberrant synaptic plasticity at glutamatergic corticostriatal synapses is believed to be a particularly critical mechanism underlying LID. As described above (2.2.2.), in rodents striatal dopamine depletion results in the loss of bidirectional synaptic plasticity and an imbalance where dMSNs exhibit only LTD while iMSNs only show LTP (Thiele et al., 2014). Subsequent levodopa treatment results in further, potentially maladaptive changes in plasticity. In *ex vivo* electrophysiological studies, the absent plasticity phenomena were restored in animals treated chronically with levodopa, possibly reflecting symptomatic

benefit; however, in dyskinetic animals the restored LTP was resistant to depotentiation (reversal) and LTD was not restored (Picconi et al., 2003; Picconi et al., 2008; Picconi et al., 2011). Therefore, LID was suggested to be caused by abnormal persistent storage of motor information (Picconi et al., 2003). In further *ex vivo* studies, the situation associated with LID was found to be essentially the opposite of the parkinsonian state: dMSNs exhibited only LTP, furthermore resistant to depotentiation, while iMSNs exhibited only LTD (Thiele et al., 2014; Shen et al., 2015). Notably, these findings are fully consistent with the classical model of overactivation of dMSNs and underactivation of iMSNs.

However, also contrasting findings have been reported. An *in vivo* study in rats found LID to be associated with abnormally persistent LTD in dMSNs and LTP that is abnormally sensitive to reversal in iMSNs (Belujon et al., 2010), findings that appear quite difficult to reconcile with the ex vivo findings or the classical model. The relative significance of aberrant indirect pathway activity in LID has also been questioned (Ko et al., 2014), and recent evidence indeed seems to point to a much more critical role for dMSNs (Girasole et al., 2018; Ryan et al., 2018). Nevertheless, at least some form of impaired bidirectional plasticity does appear to be associated with rodent LID. The cellular mechanisms of altered plasticity phenomena have been suggested to include ERK hyperactivation (Cerovic et al., 2015), hyperphosphorylation of PKA, DARPP-32, and glutamate AMPA receptors (Picconi et al., 2003; Santini et al., 2007; Santini et al., 2010), and morphological and receptor-level alterations in corticostriatal glutamatergic synapses (Suarez et al., 2016; Mellone and Gardoni, 2018). Studies in dyskinetic PD patients have provided some evidence of abnormal plasticity also in humans, showing e.g., impaired depotentiation of LTP-like synaptic facilitation in the motor cortex and the basal ganglia output nuclei (Huang, Rothwell, et al., 2011; Prescott et al., 2014).

Finally, as in the case of PD pathophysiology (see 2.2.2.), the focus of studies on basal ganglia neurotransmission in LID has begun to turn from activity rates within segregated pathways to pathologically synchronized activity. This follows in particular from the fact that further silencing of the GPi with lesioning does not provoke but alleviates LID in PD patients (Fasano et al., 2012) – a finding completely in contrast to the classical model. Recordings made in PD patients through DBS electrodes in the STN have revealed that LID is associated with reduced beta band activity (possibly in part reflecting the therapeutic effect) and, notably, increased alpha-theta band (4–10 Hz) oscillations that are coherent with cortical motor activity (Alonso-Frech et al., 2006; Rodriguez-Oroz et al., 2011). This increased synchronization is suggested to allow the release of involuntary motor sequences (Ko et al., 2014), and silencing the GPi is thought to result in beneficial disruption of the abnormal synchronicity (Guridi and Alegre, 2017). Furthermore, recent studies in patients and animal models have associated LID with neuronal activation, anatomical changes, and aberrant high-frequency oscillations in various cortical areas, along with abnormal cortex-basal ganglia connectivity (Cerasa et al., 2015; Herz et al., 2015; Swann et al., 2016; Girasole et al., 2018).

2.3.3. Current treatment options for LID and future perspectives

The current treatment options for LID remain relatively sparse and with significant drawbacks (Fox et al., 2018). Delaying levodopa treatment is a traditional but controversial approach (see 2.2.3.). DBS of the STN or the GPi typically results in significant and long-lasting improvement of both parkinsonism and LID (Moro et al., 2010; Fasano et al., 2012). The antidyskinetic effects of GPi-DBS are direct and acute, while the effects of STN-DBS are delayed and believed to be mediated through reduced levodopa dosages (Fasano et al., 2012; Bastide et al., 2015). However, DBS is typically not considered as a first-line treatment and may result in adverse effects such as cognitive decline or mood disorders (Fasano et al., 2012).

The only pharmacological treatment for LID with reasonably strong evidence for efficacy is amantadine (Fox et al., 2018). Amantadine is a glutamate NMDA receptor antagonist with a wide variety of additional targets (Blanpied et al., 2005; Bido et al., 2011). In rodents, amantadine prevents levodopa-induced GABA increases in the SNR (Bido et al., 2011), possibly reflecting inhibition of dMSNs. Some controversy has existed on the long-term efficacy of amantadine, with one controlled clinical study showing a significant antidyskinetic effect but a benefit lasting less than 8 months (Thomas et al., 2004). However, other controlled clinical studies have observed efficacy lasting for 1 year, albeit in a small population (Metman et al., 1999), and that cessation of amantadine after 0.5–1 years of treatment worsened LID (Wolf et al., 2010; Ory-Magne et al., 2014). Recently, long-term (up to 2 years) controlled trials of extended-release amantadine showed significant antidyskinetic efficacy over placebo (Elmer et al., 2018; Isaacson et al., 2018). However, the results also suggest that not all patients benefited, and furthermore adverse effects resulted in a discontinuation rate of 20 % (8 % for placebo). Thus, amantadine treatment may not suitable or beneficial for all patients. Amantadine has also been reported to have neurotoxic and genotoxic effects in mice, albeit only at high doses (Kaefer et al., 2010).

Many preclinically and even some clinically promising future treatment avenues exist (Bastide et al., 2015). Delivery of levodopa *via* continuous intrajejunal infusion, aiming at avoiding pulsatile dopamine release, was recently found to provide effective symptom relief with reduced dyskinesia that lasted for many years (Fernandez et al., 2018). However, a high frequency of adverse events such as device failures suggests that further technical development may be necessary. Other novel levodopa delivery modes have included controlled-release formulations and subcutaneous or intrapulmonary administration (Poewe and Antonini, 2015). The atypical antipsychotic clozapine may be effective against LID but has significant safety concerns (Fox et al., 2018). Reflecting the role of serotonin terminals in LID, a 5-HT1A/B autoreceptor agonist showed antidyskinetic effects in a small controlled clinical trial (Svenningsson et al., 2015). Besides amantadine, other drugs acting at glutamate receptors have been studied with mostly conflicting clinical results (Mellone and Gardoni, 2018). Some other drugs with preliminarily promising clinical results include

MAO-B inhibitors, cannabinoids, and α2 adrenergic receptor antagonists (Bastide et al., 2015). Finally, preclinical studies suggest nAChR ligands as potential antidyskinetic drugs (see 2.4.4.).

2.4. Nicotinic acetylcholine receptors in Parkinson's disease

2.4.1. Nicotinic receptor structure and function

Cholinergic innervation, utilizing acetylcholine as a neurotransmitter, covers most of the mammalian brain (Figure 6; Karczmar, 2007). The cholinergic system thus modulates neurotransmission in numerous brain areas, including the nigrostriatal dopaminergic pathway (Dani and Bertrand, 2007). Cholinergic neurotransmission is critical also in the neuromuscular junctions and autonomic ganglions of the peripheral nervous system (Mao et al., 2006; Martyn et al., 2009). Furthermore, acetylcholine and its receptors are found on numerous non-neuronal cells such as glia, endothelial cells and immune cells (Albuquerque et al., 2009). The two main types of acetylcholine receptors are the G-protein-coupled muscarinic receptors (Scarr, 2012) and nicotinic receptors, ion channel receptors consisting of five subunits (Figure 7; Albuquerque et al. 2009).

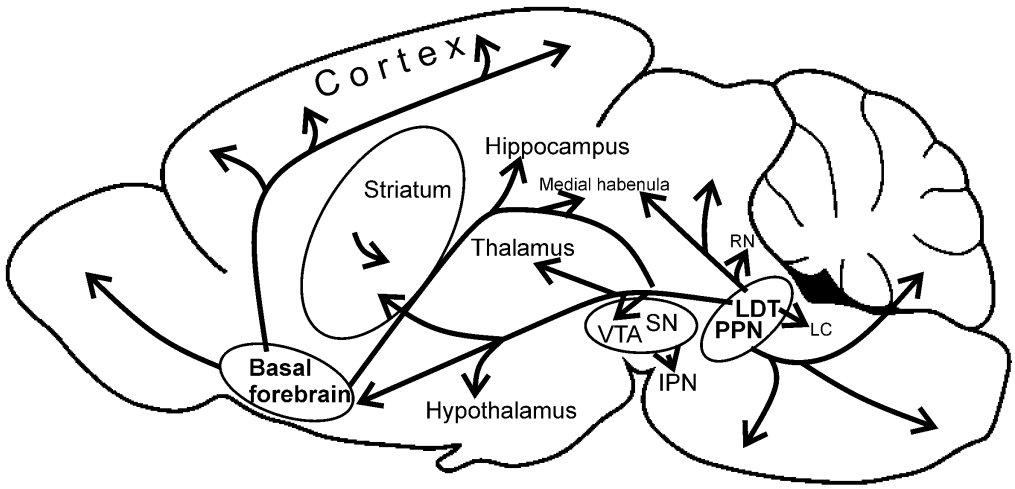

Figure 6. Cholinergic innervation in the rodent brain. Widespread innervation throughout the brain arises from cholinergic neurons located in several nuclei of the basal forebrain as well as in the pedunculopontine nucleus (PPN) and the laterodorsal tegmental nucleus (LDT) of the brainstem. In addition, local cholinergic interneurons densely innervate the striatum. Simplified representation with selected pathways and target areas shown. Anatomical accuracy approximate only. IPN = interpeduncular nucleus LC = locus coeruleus; SN = substantia nigra; RN = raphe nuclei; VTA = ventral tegmental area. Information sources: Karczmar (2007), Dautan et al. (2014)

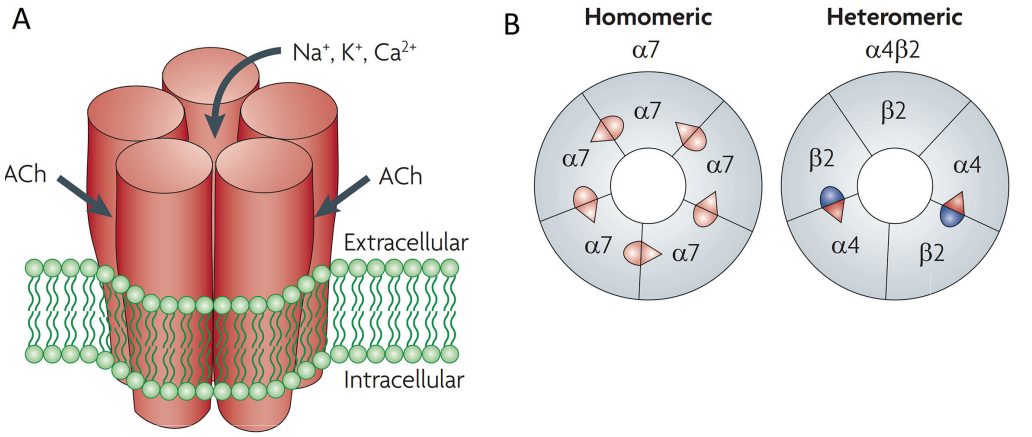

Figure 7. Nicotinic acetylcholine receptor structure. **A**: Schematic of a nicotinic receptor. Five transmembrane subunits form a central aqueous pore permeable to specific cations. Acetylcholine binding sites are located in the extracellular domain. **B**: Schematic of the pentameric structure of a nicotinic receptor. Examples of a homomeric (α7) and a heteromeric (α4β2) receptor are shown. Acetylcholine binding sites are formed at subunit interfaces. Adapted with permission from Springer Nature: Nature Reviews Neuroscience, Changeux (2010), © Springer Nature 2010

Nicotinic receptors can be either homomeric or heteromeric, with 17 different subunits (α1–α10, β1–β4, γ, δ, ε) found in vertebrates (Albuquerque et al., 2009; Millar and Gotti, 2009). Subunits α1, β1, γ, δ, and ε are expressed only in neuromuscular junctions, while the other 12 subtypes (α2–α10, β2–β4) form neuronal nAChRs expressed in the CNS and in autonomic ganglions (Albuquerque et al., 2009; Millar and Gotti, 2009). Note that also non-neuronal cells express the "neuronal" nAChR subtypes (Albuquerque et al., 2009; Millar and Gotti, 2009). Although cloned subunits are able to form a vast variety of combinations, tightly controlled transcription, assembly, and post-translational modification of native nAChRs enables the expression of only certain subunit combinations depending on the brain area and cell type (Albuquerque et al., 2009). Nevertheless, for instance Millar and Gotti (2009) list as many as 29 different receptor subtypes identified in the vertebrate nervous system. nAChR subtypes are usually referred to by their subunit composition, with an asterisk denoting the possible inclusion of other subunits (e.g., α4β2*; Millar and Gotti, 2009). The majority of neuronal nAChRs are either homomeric α7 nAChRs or heteromeric nAChRs consisting of a single type of α and β subunits, most commonly α4β2 (Millar and Gotti, 2009). However, also many diverse subtypes composed of more than two different subunits are found in numerous brain areas (Millar and Gotti, 2009). See Figure 8 for a depiction of the expression of (some of) the known nAChR subtypes in the rodent nervous system. nAChR expression in primates is less extensively studied but known to exhibit

comparable diversity, with many similarities but also some species differences in subtype expression (Zoli et al., 2015).

Acetylcholine binding sites in nAChRs are located at interfaces between an α subunit and another subunit (in neuronal nAChRs another α, β2, or β4), while subunits α5, β1, and β3 do not directly participate in ligand binding but can affect it (Albuquerque et al., 2009). After a ligand interacts with a binding site, hydrogen bond reorganization generates a rotational movement that propagates to the transmembrane domain and leads to channel opening *via* displacement of pore-blocking residues and widening of the pore (Albuquerque et al., 2009). In the neuromuscular junction this results in Na^+ influx and

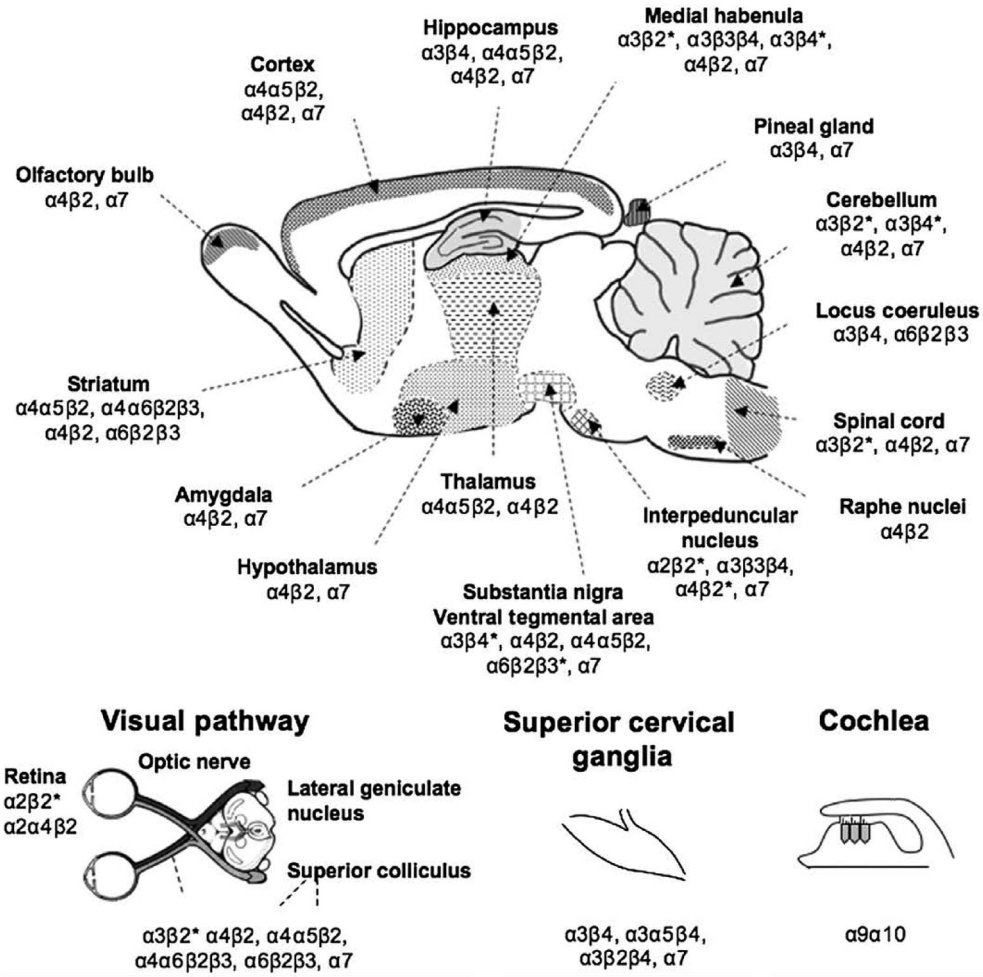

Figure 8. Diversity of neuronal nicotinic receptor expression in rodents. Receptor subtypes were collected from studies using knock-out mice and various methods to detect subunit mRNA and assembled receptors. Adapted with permission from Elsevier: Neuropharmacology, Millar and Gotti (2009), © Elsevier 2008

muscle depolarization (Martyn et al., 2009). Activation of neuronal nAChRs can also lead to Na+ influx and fast membrane depolarization; however, changes in intracellular Ca2+, through influx *via* nAChRs themselves and through activated voltage-dependent calcium channels, as well as through release from intracellular reserves, are considered more important (Dajas-Bailador and Wonnacott, 2004; Dani and Bertrand, 2007; Albuquerque et al., 2009). Neuronal nAChR stimulation can activate numerous calcium-dependent kinases and downstream transcription factors, and nAChR-mediated alterations in intracellular calcium signaling are associated with many cellular phenomena such as neurotransmitter release, gene expression, neuronal plasticity, and apoptosis (Dajas-Bailador and Wonnacott, 2004; Albuquerque et al., 2009).

A characteristic property of nAChRs is agonist-induced desensitization, leading to a state where the receptor cannot be activated despite still binding ligands with a high affinity (Picciotto et al., 2008). nAChR agonists and antagonists can therefore have similar pharmacological effects and, conversely, a nAChR agonist can have variable and even simultaneous agonist and antagonist effects (Picciotto et al., 2008). Another typical property of nAChRs is altered receptor expression (up- or downregulation) in response to chronic agonist treatment (Picciotto et al., 2008; Albuquerque et al., 2009). Both desensitization kinetics and the amount and direction of expression changes depend on the subunit composition of the receptor as well as issues such as ligand concentration and duration of exposure (Picciotto et al., 2008).

Both somatodendritic (postsynaptic) and presynaptic nAChRs exist, and a significant part of nAChR-mediated neurotransmission is also suggested to be non-synaptic volume transmission (Dani and Bertrand, 2007; Albuquerque et al., 2009). Somatodendritic nAChRs mediate fast excitatory neurotransmission mainly in neuromuscular junctions and autonomic ganglions (Mao et al., 2006; Martyn et al., 2009), although they are also found in restricted CNS locations such as in the hippocampus and the substantia nigra (Albuquerque et al., 2009; see also next section). The main function of nAChRs in the CNS, however, is thought to be the modulation of neurotransmitter release, mediated by presynaptic nAChRs located on nerve terminals. Presynaptic nAChRs stimulate or enhance the release of neurotransmitters *via* elevation of the intracellular calcium concentration and calcium-dependent exocytosis (Dajas-Bailador and Wonnacott, 2004; Albuquerque et al., 2009). Note that also axonal and "preterminal" nAChRs that modulate action potential-dependent neurotransmission exist (Albuquerque et al., 2009). As cholinergic neurons innervate most of the brain, and neuronal nAChRs modulate the release of most neurotransmitters (Dani and Bertrand, 2007), disruption of nAChR-mediated neurotransmission has been associated with numerous disorders (Taly et al., 2009). Accordingly, nAChR ligands have been suggested as possible treatments for many diseases such as Parkinson's disease, Alzheimer's disease, nicotine addiction, depression, and attention disorders (Taly et al., 2009).

2.4.2. Nicotinic receptor-mediated neuromodulation in the basal ganglia

Perhaps the most studied example of nAChR-mediated neuromodulation is the regulation of basal ganglia neurotransmission. In particular, the complex regulation of midbrain dopaminergic neuron activity and the striatal release of dopamine and other neurotransmitters by nAChRs has been extensively studied, and involves numerous nAChR subtypes located on different neurons (see Figure 9; Quik and Wonnacott, 2011). Electrophysiological evidence suggests the expression of somatodendritic α6β2*, α4(non-α6)β2*, and α7 nAChRs on dopaminergic neurons of the mouse SNC, with some heteromeric receptors possibly also including the α5 subunit (Klink et al., 2001). Immunoprecipitation studies in 6-OHDA-lesioned rats further suggest that the heteromeric somatodendritic nAChRs may include α4β2, α2α4β2, α4α5β2, α4β2β3, and α4α6β2β3 subtypes (Gotti et al., 2010). However, the SN and the VTA were not separated, and their somatodendritic dopaminergic neuron nAChRs do differ (Klink et al., 2001; Keath et al., 2007). Some of these subtypes could also be only destined for trafficking to terminal areas.

While there is no evidence of postsynaptic nAChRs on striatal MSNs (Quik and Wonnacott, 2011), nAChRs located on striatal dopamine terminals indirectly modulate MSN activity. Immunoprecipitation experiments in nAChR subunit-knockout mice and 6-OHDA-lesioned rats suggest the expression of α4β2, α4α5β2, α4β2β3, α6β2β3, and α4α6β2β3 nAChRs on striatal dopamine terminals (Champtiaux et al., 2003; Gotti et al., 2010).

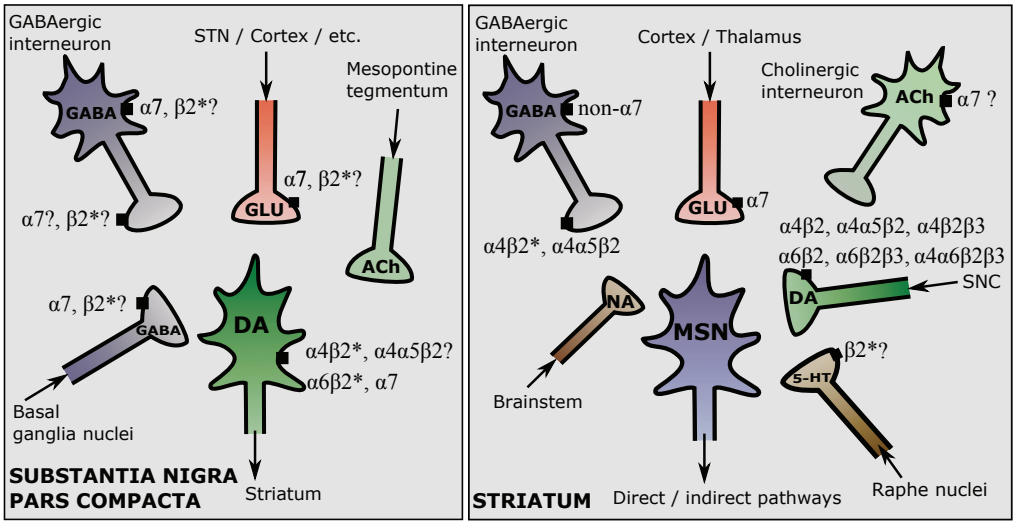

Figure 9. Suggested subtypes and neuronal localization of nicotinic receptors contributing to neuromodulation in the rodent basal ganglia. nAChR subtypes modulating neurotransmitter release and local interneuron activity in the substantia nigra pars compacta (SNC, left) and the striatum (right) are shown. The positioning of the figure elements does not reflect anatomical proportions. ACh = acetylcholine; DA = dopamine; GABA = γ-aminobutyric acid; GLU = glutamate; NA = noradrenaline; STN = subthalamic nucleus; 5-HT = 5-hydroxytryptamine (serotonin)

Knockout mice studies have shown that dopamine release from striatal synaptosomes is modulated largely by the same nAChR subtypes, although a role was suggested also for α6β2 nAChRs and a contribution by α4β2β3 nAChRs could not be confirmed (Champtiaux et al., 2003; Salminen et al., 2004; Salminen et al., 2007). A particularly noteworthy nAChR population are the α6 subunit-containing (α6*) nAChRs, which in rodents are selectively expressed in dopaminergic and noradrenergic nuclei (Le Novère et al., 1996) and, in the striatum, on dopamine terminals (Quik, Sum, et al., 2003). This expression profile suggests that α6* nAChRs might be used for selective targeting of dopaminergic neurons in e.g., Parkinson's disease. Immunoprecipitation and functional studies agree that the α4α6β2β3 subtype comprises the majority of dopamine terminal α6* nAChRs, and it is also the most sensitive to agonist activation (Salminen et al., 2007; Gotti et al., 2010). An invaluable tool in the study of α6* nAChRs has been α-conotoxin MII (CtxMII), an α3* and α6* nAChR antagonist that in rodent dopaminergic areas can be used to selectively target α6* nAChRs (Champtiaux et al., 2002). Another intriguing nAChR population are those containing the α5 subunit (α5* nAChRs), which are discussed in more detail below (2.4.3.).

In general, cholinergic input from the mesopontine tegmentum to the SNC (and VTA) has been suggested to be a critical regulator of brain dopaminergic systems (Maskos, 2008), while striatal dopamine terminal nAChRs have been suggested to act as a presynaptic "filter" dynamically regulating dopamine release (Exley and Cragg, 2008). Rodent brain slice studies suggest that acetylcholine released by cholinergic interneurons not only modulates but is essential for striatal dopamine release (Zhou et al., 2001) and can even generate dopamine release directly, independently of the activity of the dopaminergic neuron (Threlfell et al., 2012). Cholinergic interneurons in turn are engaged in particular by thalamostriatal projections, which thus indirectly regulate dopamine release *via* nAChRs and possibly modulate corticostriatal input in response to salient stimuli (Ding et al., 2010; Threlfell et al., 2012). Thus, while the firing rate and pattern of a nigrostriatal neuron is usually considered the primary regulator of striatal dopamine release (Quik and Wonnacott, 2011), the participation by presynaptic nAChRs is also of significant importance. Findings in rodent brain slices also suggest that in conditions where at least some circuit effects remain intact, α6β2* nAChRs (in particular α4α6β2β3) have a critical role in the ventral striatum, while α4α5β2 nAChRs dominate the control of dopamine release in the dorsal striatum (Exley and Cragg, 2008; Exley et al., 2012). Note also that at least in brain slice preparations exogenous nAChR agonists typically act *via* desensitization (i.e., similarly as antagonists; Zhou et al., 2001).

Besides dopaminergic neurons, nAChRs also modulate the activity and neurotransmitter release of many other neurons. In the SNC, functional evidence suggests the existence of α7 and non-α7 (possibly β2*) nAChRs on glutamate and GABA terminals as well as somatodendritically on GABAergic interneurons, all of which can indirectly modulate the activity of dopaminergic neurons (Keath et al., 2007; Quik and Wonnacott, 2011). In the striatum, presynaptic α7 nAChRs on glutamate terminals (Kaiser and Wonnacott, 2000),

α4β2 and α4α5β2 nAChRs on GABA terminals (McClure-Begley et al., 2009), and nAChRs of an unknown subtype (possibly β2*) on raphestriatal serotonin terminals (Schwartz et al., 1984; Reuben and Clarke, 2000) regulate neurotransmitter release and thus can indirectly modulate dopamine release as well as MSN activity. Some striatal GABAergic interneurons also express somatodendritic nAChRs of an unknown non-α7 subtype (Koos and Tepper, 2002). Some studies have also found evidence of somatodendritic nAChRs (possibly α7) on cholinergic interneurons (Sandor et al., 1991; Xiao et al., 2009). Finally, nAChR-mediated modulation in other basal ganglia areas than those already discussed has been little studied, but based on mRNA expression, nAChRs are expressed at least in the globus pallidus and the STN (Wada et al., 1989). In addition, somatodendritic nAChRs have been shown to modulate the activity of the GABAergic projection neurons of the SNR (Klink et al., 2001).

The nAChR subtypes modulating dopaminergic neurotransmission in primates appear mostly similar to those in rodents, although some differences also exist (see Table 1). Studies on the effects of MPTP lesioning on mRNA and ligand binding suggest that similar to rodents, dopaminergic neurons of the monkey SNC express both α3/α6* and non-α3/α6 somatodendritic nAChRs, but unlike rodents α7 nAChRs may be restricted to non-dopaminergic neurons (Quik et al., 2000; Quik et al., 2002). The nAChR repertoire of the monkey striatum has been revealed in more detail in a series of ligand binding, immunoprecipitation, and MPTP lesioning studies (Quik et al., 2005). In brief, the monkey striatum was found to express α3β2*, α4β2*, α6β2*, α2β2* and α7 nAChRs but no α5 or β4 subunits, with evidence further suggesting that α3β2*, α6β2β3, and α4α6β2β3 nAChRs are located on dopamine terminals, α4β2 nAChRs are located on both dopamine terminals and non-dopaminergic neurons, and α2β2* and α7 nAChRs are located only on non-dopaminergic neurons. Note the selective localization of α6* nAChRs on dopamine terminals, similar to rodents. A notable difference to rodents is that α3β2* nAChRs are expressed in the monkey striatum and – perhaps consequently – α3/α6* (CtxMII-sensitive) receptors make up a much higher proportion of striatal nAChRs (Quik, Sum, et al., 2003) and have a more dominant role in the regulation of dopamine release (Mccallum et al., 2005; Perez et al., 2009).

While high amounts of nAChRs are present in the human SNC (Gotti et al., 1997), their composition has not been studied in detail (Zoli et al., 2015). In the human striatum, Western blot protein measurements have detected α2–α7 and β2–β3 nAChR subunits, but no changes associated with PD (i.e., dopamine terminal loss) were found barring a small decrease in α3 in one study (Martin-Ruiz et al., 2002; Guan et al., 2002). In contrast, experiments combining ligand binding and immunoprecipitation (reflecting assembled receptors) found only α4, α6, β2, and β3 subunits (i.e., no α3) at levels above the detection limit (Gotti et al., 2006; α7 was not studied). Analyses of tissue from PD patients suggest that similar to other species, human striatal α6β2* nAChRs (many of which contain also α4 and/or β3) are selectively expressed on dopamine terminals while α4(non-α6)β2* nAChRs are expressed both on dopaminergic and non-dopaminergic sites (Quik et al., 2004;

Gotti et al., 2006). Interestingly, the proportion of α3/α6* (CtxMII) binding sites in the human striatum resembles that of rodents instead of monkeys (Quik et al., 2004), further suggesting that striatal α3* nAChRs may in fact be unique to monkeys.

Table 1. Nicotinic receptor subtypes suggested to be expressed on dopaminergic and other neurons in the rodent, monkey and human substantia nigra pars compacta (SNC) and striatum. The table is based on the evidence reviewed above.

	Rodent	**Monkey**	**Human**
SNC: Dopaminergic neurons	α4β2*, α4α5β2? α6β2* α7	α3/α6* non-α3/α6	?
SNC: Other neurons	β2* α7	β2*? α7?	?
Striatum: Dopamine terminals	α4β2, α4α5β2, α4β2β3 α6β2, α6β2β3, α4α6β2β3	α3β2* α4β2 α6β2β3, α4α6β2β3	α4β2 α6β2β3, α4α6β2β3
Striatum: Other neurons	α4β2*, α4α5β2 α7	α2β2*, α4β2 α7	α4β2* α7?

2.4.3. α5 nicotinic receptors*

A major part of the present studies focused on nAChRs containing the α5 subunit. Cloned α5 subunits have been shown to form functional nAChRs but only when co-expressed with both a different α subunit and a β subunit (Ramirez-Latorre et al., 1996; Wang et al., 1996). Unlike other α subunits, the α5 subunit thus does not participate in agonist binding. Nevertheless, the presence of an α5 subunit has significant effects on ligand binding and receptor function. In cloned nAChRs, α5 incorporation results in faster and more extensive acute desensitization, increased Ca^{2+} permeability and, depending on the other subunits, either increased or decreased agonist affinity (Ramirez-Latorre et al., 1996; Wang et al., 1996; Gerzanich et al., 1998; Tapia et al., 2007; Kuryatov et al., 2008). *Ex vivo* rodent studies suggest that α5 incorporation into a native nAChR results in increased receptor function (Brown et al., 2007), resistance to upregulation (Mao et al., 2008), and a decreased sensitivity to short-term desensitization with a faster recovery but no change in desensitization extent (Grady et al., 2012); note the complex and in part contradictory effects on desensitization kinetics in cloned and native nAChRs.

In vertebrates, α5* nAChRs are expressed both in peripheral ganglions and in the CNS (Gerzanich et al., 1998). As many as a third of human neocortical α4β2 nAChRs and 20 % of rat brain α4β2 nAChRs may contain an α5 subunit (Gerzanich et al., 1998). However, α5* nAChRs show a relatively limited localization. In rats, α5 mRNA is expressed at relatively high levels in parts of the hippocampus, the interpeduncular nucleus, the

SNC, and the VTA, and at moderate levels in brain areas such as the isocortex, olfactory nucleus, and a few others (Wada et al., 1990). Immunoprecipitation studies in rodents have detected assembled α5* nAChRs in a wider array of brain areas (but always as a minority of heteromeric nAChRs): the hippocampus, interpeduncular nucleus, medial habenula, hypothalamus, cortex, striatum, thalamus, the olfactory tubercle, and the superior colliculus; the SN and the VTA were not studied (Brown et al., 2007; Mao et al., 2008). At least in the rat hippocampus, striatum, cortex, and thalamus, virtually all α5* nAChRs are of the α4α5β2 subtype (Mao et al., 2008).

α5* nAChRs have been found to contribute to GABA release in the striatum, cortex, and hippocampus (McClure-Begley et al., 2009) and to the activity of serotonergic neurons of the dorsal raphe nucleus (Besson et al., 2016). Most importantly for the present thesis, α5* nAChRs have a significant role in the modulation dopaminergic neurotransmission, at least in rodents. In the SNC, α5 subunit mRNA is expressed in 80 % or more of dopaminergic neurons, and their somatodendritic nAChRs may include α5* nAChRs (Klink et al., 2001). Immunoprecipitation and dopaminergic lesion studies suggest that a third of all heteromeric nAChRs in midbrain dopaminergic neurons consist of α4α5β2 nAChRs (Gotti et al., 2010), and that in the striatum α4α5β2 nAChRs make up as many as 40–50 % of nAChRs on dopamine terminals (Gotti et al., 2010). Similarly, genetic deletion of the α5 subunit results in a ca. 60 % decrease in nAChR-mediated dopamine release from striatal synaptosomes (Salminen et al., 2004; Grady et al., 2010). As already mentioned, nAChR-mediated control of dopamine release in the dorsal striatum was also found to critically depend on α4α5β2 nAChRs in brain slice voltammetry studies (Exley et al., 2012). Furthermore, in dopaminergic neurons of the ventral midbrain the α5 subunit helps maintain α4* nAChR expression and enhances α4*-mediated currents (Chatterjee et al., 2013) and is essential for elevation of intracellular Ca^{2+} concentrations mediated by heteromeric nAChRs (Sciaccaluga et al., 2015). However, note that α5* nAChRs are not known to be present on striatal dopamine terminals in primates (see the previous section and 6.2.2. below).

Mice lacking the α5 subunit do not exhibit obvious physical or neurological deficits and are normal in various measures of autonomic function, behavior, brain anatomy, and expression of other nAChR subunits (Wang et al., 2002; Salas et al., 2003). However, they do show reduced sensitivity to many effects of nicotine (Salas et al., 2003; Jackson et al., 2010), including those associated with nicotine addiction. More specifically, mice lacking the α5 subunit exhibit increased nicotine self-administration and reduced somatic signs of nicotine withdrawal (Salas et al., 2009; Jackson et al., 2010; Fowler et al., 2011; Morel et al., 2014). As their mesolimbic pathway furthermore shows decreased sensitivity to nicotine, the behavioral findings have been suggested to reflect decreased sensitivity to nicotine reward (Morel et al., 2014; Besson et al., 2016). α5* nAChRs of the habenulo-interpeduncular pathway have also been suggested to be important, and might mediate aversion to nicotine and thus act to inhibit nicotine intake (Salas et al., 2009; Fowler et al., 2011; Morton et al., 2018). Importantly,

many human studies have associated polymorphisms in the α5 subunit gene *CHRNA*5 with nicotine dependence, heavy smoking, and lung cancer (Bierut et al., 2008; Thorgeirsson et al., 2008; Weiss et al., 2008; Hung et al., 2008). Rodent studies suggest that the most studied variant (D398N) results in a loss of receptor function (Morel et al., 2014; Sciaccaluga et al., 2015), which has been suggested to lead to increased nicotine consumption either due to reduced sensitivity of dopaminergic reward systems (Morel et al., 2014) or due to reduced sensitivity to nicotine aversion (Fowler et al., 2011; Morton et al., 2018).

Behavioral studies also suggest α5* nAChRs may have important roles in attention (Bailey et al., 2010) and anxiety (Besson et al., 2016). The findings of an important role in dopaminergic neurotransmission might also give reason to suspect a role for α5* nAChRs in Parkinson's disease and its treatment; however, they have not previously been studied in the context of PD.

2.4.4. Nicotinic receptors as drug targets in Parkinson's disease

Changes in nicotinic receptors in Parkinson's disease

Many studies on post mortem tissue from PD patients have found significant reductions in nicotinic receptor levels in various brain areas including the SNC, striatum, hippocampus, thalamus, and cortex (e.g., Rinne et al., 1991; Aubert et al., 1992; Perry et al., 1995). As mentioned above, experiments on human striatal tissue suggest that the nAChR subtypes which are preferentially lost in PD (i.e., expressed on dopamine terminals) include both α6β2* and α4(non-α6)β2* nAChRs, the former in particular being expressed only on dopaminergic neurons (Quik et al., 2004; Gotti et al., 2006). Note, however, also the contrasting findings of selectively decreased α3 subunit levels (Guan et al., 2002) or no reduction in nAChR subunit levels (Martin-Ruiz et al., 2002). Interestingly, levels of the α7 nAChR, not expressed on dopamine terminals, have been reported to be increased in the temporal cortex and caudate of PD patients (Guan et al., 2002; Morissette et al., 2016). Studies in monkey and rodent models of dopaminergic neurodegeneration have yielded similar results, observing near-total striatal losses of α6β2* nAChRs (and/or α3β2* in monkeys), less pronounced striatal losses of α4(non-α6)β2* nAChRs, and unaffected or increased striatal α7 nAChR levels (Quik, Sum, et al., 2003; Kulak and Schneider, 2004; Quik et al., 2005; Quik, Campos, and Grady, 2013). Animal model studies further suggest that the α4α6β2β3 nAChR population may be particularly vulnerable to nigrostriatal damage (Bordia et al., 2007). In summary, consistent evidence across species shows that the loss of striatal dopamine terminals in PD results in a selective loss of associated nAChR populations, in particular those containing the α6 subunit.

Nicotinic receptors and dopaminergic neuroprotection

Evidence for nAChR-mediated beneficial effects includes numerous epidemiological studies as well as extensive preclinical investigations both *in vitro* and *in vivo*. Here, only dopaminergic neuroprotection related to PD will be discussed; for general reviews on nAChR-mediated neuroprotection, see e.g., Mudò, Belluardo, and Fuxe (2007), Picciotto

and Zoli (2008), or Posadas et al. (2013). Also nAChR-mediated pro-cognitive effects and protection against Alzheimer's disease (Mudò, Belluardo, and Fuxe, 2007; Picciotto and Zoli, 2008; Quik et al., 2015) should be mentioned, as they may be relevant for instance regarding PD-associated cognitive decline.

A negative correlation between tobacco smoking and PD has been observed in dozens of epidemiological studies, conducted during the past 50+ years by different investigators in different countries. Systematic reviews and meta-analyses have obtained consistent results of reduced PD risk, in current smokers as well as less strongly in former smokers, that cannot be explained by confounding variables or biases such as selection bias, selective mortality of smokers, or changes in smoking behavior after diagnosis (Morens et al., 1995; Allam et al., 2004; Ritz et al., 2007; Li et al., 2015). Taken together, the risk for PD appears to be at least roughly halved in current smokers. Strong evidence for a real, biological protective effect includes an inverse dose-relationship between smoking and PD risk, with a longer and more intensive smoking history associated with a lower PD risk (Gorell et al., 1999; Thacker et al., 2007; Chen et al., 2010); changes in PD incidence mirroring changes in smoking prevalence (Morozova et al., 2008); reduced PD risk in passive smokers (Searles Nielsen et al., 2012); twin studies ruling out genetic variability as a confounder (Tanner et al., 2002; Wirdefeldt et al., 2005); and findings that the lower PD risk is independent of personality traits such as lower sensation seeking or neuroticism (Evans et al., 2006; Sieurin et al., 2016). The use of cigars, pipes, and chewing tobacco is also inversely associated with PD (O'Reilly et al., 2005; Ritz et al., 2007). Note, however, that some contrasting evidence does exist; for instance, one study found no association of the progression of parkinsonism, cognitive impairment, or mood with cigarette smoking (Alves et al., 2004). A recent study also found that PD patients are able to quit smoking more easily than controls, possibly suggesting that instead of a neuroprotective effect, a reduction in nicotine reward leads to easier quitting in individuals at risk for PD (Ritz et al., 2014). In totality, however, the evidence for some form of a protective effect is convincing and widely accepted. Smoking, however, can obviously not be recommended due to its numerous and well-known adverse effects. Thus, the identification of the protective constituent and its biological targets is of paramount importance. MAO-B inhibitors present in tobacco smoke are one possibility (Castagnoli and Murugesan, 2004). Another, much more studied possibility is nicotine, the primary psychoactive component of tobacco.

Extensive *in vitro* evidence has shown neuroprotective effects by nicotine and other nAChR ligands in neuronal cell lines as well as rat and mouse cortical, hippocampal, and striatal cultures against diverse neurotoxic insults such as amyloid-β, glutamatergic excitotoxicity, kainic acid, growth factor deprivation, oxygen deprivation, and oxidative stress (Mudò, Belluardo, and Fuxe, 2007; Picciotto and Zoli, 2008; Quik et al., 2015). Furthermore, a number of studies in animal models have described nAChR-mediated neuroprotection against ischemic and lesion insults in the hippocampus and the neocortex (Mudò, Belluardo, and Fuxe, 2007). In the case of dopaminergic neurons, *in vitro* studies

in rodent dopaminergic neuron cultures have shown nicotine to partially protect against neurotoxicity induced by lipopolysaccharide, rotenone, or the toxic MPTP metabolite 1-methyl-4-phenylpyridinium (MPP^+), as well as against spontaneous degeneration (Jeyarasasingam et al., 2002; Park et al., 2007; Takeuchi et al., 2009; Toulorge et al., 2011).

The most extensive investigations have focused on *in vivo* assessment of nigrostriatal neuroprotection in parkinsonian animal models. The main findings of these studies are presented in Table 2. In summary, neuroprotective effects by nicotine have been demonstrated in a number of monkey and rodent models of dopaminergic denervation and in studies utilizing many different routes and regimes of administration. Remarkably, in some studies only a few injections given before and after 6-OHDA lesioning were found to partially block striatal dopamine loss. A few studies have also found neuroprotective effects by selective α7 nAChR agonists. However, a number of studies in rodents (particularly in mice) also observed no effect or even increased denervation in nicotine-treated animals. Moreover, primate studies are rare and have observed moderate inhibition of striatal denervation but no sparing of SNC dopaminergic neurons. Whether neurotoxicity in rodents was decreased, unchanged, or increased by nicotine treatment does not appear to be clearly related to any single issue such as the lesion model or the method or timing of nicotine administration. However, the only study directly comparing pre- and post-treatments found only pretreatment to be effective (Huang et al., 2009). Furthermore, one rat study found that neuroprotective effects by nicotine were lost when the dosage was raised high enough (Ryan et al., 2001). Somewhat surprisingly, while in rats both intermittent nicotine injections and continuous treatment *via* subcutaneous minipumps had protective effects, in mice two of the three studies utilizing minipump administration showed increased neurotoxicity instead.

Both heteromeric (at least α4β2*) and homomeric (α7) nAChRs are involved in hippocampal and cortical neuroprotective effects of nicotinic agonists (Mudò, Belluardo, and Fuxe, 2007; Picciotto and Zoli, 2008; Posadas et al., 2013; Quik et al., 2015), and the same appears to be true for dopaminergic neuroprotection. Dopaminergic neuroprotection by nicotine was blocked *in vivo* by a non-selective nAChR antagonist (Costa et al., 2001) and α4 gene deletion (Ryan et al., 2001), and *in vitro* by antagonism of heteromeric nAChRs (Takeuchi et al., 2009) but not by α7 nAChR antagonism (Jeyarasasingam et al., 2002), suggesting that the neuroprotection was mediated by heteromeric (possibly α4β2*) nAChRs. On the other hand, also α7 nAChRs are involved, as demonstrated by neuroprotection by selective α7 agonists *in vivo* (Suzuki et al., 2013; Stuckenholz et al., 2013; Bordia, McGregor, Papke, et al., 2015) as well as by blockade of neuroprotection by α7 inhibition both *in vivo* (Liu et al., 2012; Suzuki et al., 2013) and *in vitro* (Park et al., 2007; Takeuchi et al., 2009; Toulorge et al., 2011). Simultaneous activation of both subtypes may be required, as nicotine but neither selective α4β2 nor α7 nAChR ligands had a neuroprotective effect in one study (Visanji et al., 2006). Finally, one study in rats observed that striatal α4α6β2* nAChRs were only expressed in association with neuroprotection by nicotine, suggesting that they may be an important mediator (Huang et al., 2009). The possible role of α5* nAChRs has not been studied.

Table 2. Studies on neuroprotection by nicotinic receptor agonists carried out in monkey, rat, and mouse models. Striatal and/or nigral denervation was measured using various methods; see the references for more details on methodology and outcomes. MFB = medial forebrain bundle; SN = substantia nigra; PAM = positive allosteric modulator

Study	Model	Drug, administration route, and duration	Inhibition of denervation Striatum	SN
Quik, Chen, et al., 2006; Quik, Parameswaran, et al., 2006	Monkey, MPTP	Nicotine, drinking water, 6 mths before + 6 mths after lesioning	Partial	None
Huang et al., 2009	Monkey, MPTP	Nicotine, drinking water, for 2 mths after lesioning	None	
Janson, Fuxe, Agnati, et al., 1988	Rat, mechanical lesion	Nicotine, minipump, for 2 wks after lesioning	Partial	Partial
Fuxe et al., 1990	Rat, mechanical lesion	Nicotine, minipump, for 2 wks after lesioning	Partial	Partial
Janson and Møller, 1993	Rat, mechanical lesion	Nicotine, minipump, for 2 wks after lesioning		Partial
Blum et al., 1996	Rat, 6-OHDA (SN)	Nicotine, minipump, for 1 wk before + 1 wk after lesioning	None	
Ryan et al., 2001	Rat, 6-OHDA (striatum)	Nicotine, minipump, for 1 wk before + 1 wk after lesioning	Full	
Bordia, McGregor, Papke, et al., 2015	Rat, 6-OHDA (MFB)	Nicotine, minipump, for 2 wks before + 8 wks after lesioning α7 agonist, similar administration	Partial Partial	
Huang et al., 2009	Rat, 6-OHDA (MFB)	Nicotine, drinking water, for 3 wks before lesioning Nicotine, drinking water, for 2 wks after lesioning	Partial None	
Costa et al., 2001	Rat, 6-OHDA (SN)	Nicotine, injection, 1 x before + 3 x after lesioning	Partial	None
Soto-Otero et al., 2002	Rat, 6-OHDA (striatum) Rat, 6-OHDA (ventricle)	Nicotine, injection, 3 x before + 4 x after lesioning Nicotine, similar administration	None Partial	
Visanji et al., 2006	Rat, 6-OHDA (MFB)	Nicotine, injection, 2 x before + 1/d for 2 wks after lesioning α4β2 agonist, similar administration α7 agonist, similar administration	Partial None None	
Yanagida et al., 2008	Rat, 6-OHDA (SN)	Nicotine, intracerebrally together with 6-OHDA nAChR PAM, similar administration Nicotine + nAChR PAM, similar administration	Partial Partial Synergistic effect	
Suzuki et al., 2013	Rat, 6-OHDA (MFB)	α7 agonist, intracerebrally together with 6-OHDA		Partial
Maggio et al., 1997	Rat, methamphetamine	Nicotine, injection, 3 x during lesioning		Full
Park et al., 2007	Rat, lipopolysaccharide	Nicotine, injection, for 4 wks before lesioning		Partial

Table 2 cont.

Study	Model	Drug, administration route, and duration	Inhibition of denervation Striatum	SN
Carr and Rowell, 1990	Mouse, MPTP	Cigarette smoke, 2/d for 5 wks before + 1 wk after lesioning	Partial	
Shahi et al., 1991	Mouse, MPTP	Cigarette smoke, 4/d for 2 wks before + 1 wk after lesioning	Partial	
Parain et al., 2003	Mouse, MPTP	Cigarette smoke, 5/d for 1 wk before + 3 wks after lesioning Nicotine, injection, similar administration	None None	Partial Partial
Janson, Fuxe, Sundström, et al., 1988	Mouse, MPTP	Nicotine, 4 injections + minipump for 2 wks after lesioning	Partial	Partial
Maggio et al., 1997	Mouse, MPTP Mouse, methamph.	Nicotine, injection, 3 x during lesioning		Partial Full
Gao et al., 1998	Mouse, MPTP	Nicotine, injection, 4/d for 11 d before + 11 d after lesioning	Partial	
Parain et al., 2001	Mouse, MPTP	Nicotine, injection, 5/d for 1 wk before + 3 wks after lesioning	Partial	Partial
Park et al., 2007	Mouse, MPTP	Nicotine, injection, 2/d for 2 wks before lesioning		Partial
Liu et al., 2012	Mouse, MPTP	Nicotine, injection, 5/d for 1 wk before + 1 wk after lesioning		Full
Stuckenholz et al., 2013	Mouse, MPTP	α7 agonist, injection, 1/d for 1 d before + 1 wk after lesioning	Partial	Partial
Ryan et al., 2001	Mouse, methamph.	Nicotine, injection, 3 x during lesioning	Full	
Khwaja et al., 2006	Mouse, paraquat	Nicotine, drinking water, 3 wks before + 4 wks during lesioning	Partial	Partial
Takeuchi et al., 2009	Mouse, rotenone	Nicotine, injection, before each lesioning dose (28 days)		Full
Perry et al., 1987	Mouse, MPTP	Cigarette smoke, 2/d for 1 wk before + 5 d after lesioning	None	
Sershen et al., 1988	Mouse, MPTP	Nicotine, drinking water, for 16 wks after lesioning	None	
Fung et al., 1991	Mouse, MPTP	Nicotine, injection, 2/d for 2 wks before lesioning	None	
Janson et al., 1992	Mouse, MPTP	Nicotine, injection, 4 x during lesioning Nicotine, minipump, for 2 wks after lesioning	Partial Toxicity increased	Partial
Behmand and Harik, 1992	Mouse, MPTP	Nicotine, minipump, for 1 wk before + 1 wk after lesioning	Toxicity increased	
Hadjiconstantinou et al., 1994	Mouse, MPTP	Nicotine, injection, 2/d for 2 wks before + 1 wk during lesioning	Toxicity increased	
Ferger et al., 1998	Mouse, MPTP	Nicotine, injection, 2/d for 1 wk before + 1 wk after lesioning	Toxicity increased	

The detailed mechanisms of nAChR-mediated neuroprotection are far from well-understood. As mentioned above, long-term nicotine treatment in monkeys resulted in moderate sparing of striatal markers of dopamine terminal integrity such as the dopamine-synthesizing enzyme tyrosine hydroxylase (TH), DAT, and dopamine levels (~20 % more remaining when compared to control animals), but no sparing of dopaminergic neurons in the SNC (Quik, Parameswaran, et al., 2006). However, despite the modest neuroprotective effects, aberrant striatal functions observed in the MPTP-treated monkeys, such as enhanced *ex vivo* dopamine release and turnover as well as a loss of LTD, were almost fully normalized in nicotine-treated animals (Quik, Chen, et al., 2006). Chronic nicotine treatment also upregulated striatal α4β2* nAChRs and protected against MPTP-induced α4β2* nAChR loss, while downregulating a subset of striatal α6β2* nAChRs but restoring a subset that were lost due to MPTP (Bordia et al., 2006). Thus, rather than exerting direct protection against nigrostriatal neuron loss, nicotine treatment may allow more efficient compensation against the resulting striatal dopaminergic dysfunction and nAChR loss. Note that many rodent studies also measured only striatal markers (Table 2), such as TH or dopamine, making it unclear whether compensation or actual sparing of dopaminergic neurons had occurred.

As for actual neuroprotection, alterations in intracellular calcium signaling are often proposed as a mechanism, given that nAChR activation typically results in Ca^{2+} influx and/or mobilization (Mudò, Belluardo, and Fuxe, 2007; Picciotto and Zoli, 2008). nAChR activation could lead to modest increases in intracellular calcium that might confer protection against larger calcium influxes resulting from e.g., excitotoxicity (Picciotto and Zoli, 2008). In cultured rat dopaminergic neurons, nicotine-induced protection against spontaneous neurodegeneration was indeed found to depend on the elevation of cytosolic Ca^{2+} levels, possibly resulting in the activation of a calcium-mediated prosurvival pathway (Toulorge et al., 2011). Other suggested mechanisms relate to oxidative stress and mitochondrial dysfunction. Nicotine can both have antioxidant properties and induce oxidative stress (Newman et al., 2002), and while some *in vitro* studies have found nicotine to have radical scavenging properties (Ferger et al., 1998), others have not observed radical scavenging but rather inhibition of 6-OHDA-induced lipid peroxidation (Soto-Otero et al., 2002). Nicotine has also been shown to protect rat brain mitochondria against various damaging insults both *in vitro* and *in vivo* (Cormier et al., 2003; Xie et al., 2005). Intriguingly, mitochondria themselves have been shown to express various nAChR subtypes (Lykhmus et al., 2014).

Another often suggested mechanism for nicotine's neuroprotective effects is nAChR-mediated stimulation of neuroprotective or neurotrophic growth factors (Mudò, Belluardo, and Fuxe, 2007; Picciotto and Zoli, 2008). In rodents, nAChR agonists increase the expression of various growth factors such as the nerve growth factor, the fibroblast growth factor-2, and the BDNF, along with their tyrosine kinase A and B receptors (Maggio et al., 1997; French et al., 1999; Belluardo et al., 2000; Jonnala et al., 2002; Formaggio et al., 2010).

Nicotine treatment was also found to enhance neuronal precursor cell proliferation in the rat brain (Mudò, Belluardo, Mauro, et al., 2007). nAChRs on non-neuronal cells, such as immune cells, could also be involved in neuroprotection. Indeed, neuroprotective effects by nAChR agonists in rodent models have been found to be associated with modulation of the activation of microglia and astrocytes and attenuation of brain inflammation (Park et al., 2007; Liu et al., 2012; Stuckenholz et al., 2013). Finally, an interesting novel hypothesis suggests that smoking-induced changes in the gut microbiota could mitigate intestinal inflammation and attenuate α-synuclein misfolding and propagation originating in the enteric nervous system (Derkinderen et al., 2014).

Nicotinic receptors and levodopa-induced dyskinesia

Nicotine has been observed to have little or no antiparkinsonian effect in preclinical monkey and rodent models, although it may potentiate some behavioral effects of levodopa (Domino et al., 1999; Quik et al., 2007; Bordia et al., 2008; Huang, Grady, and Quik, 2011). Clinical studies on the treatment of PD motor symptoms with nAChR agonists have also been unconvincing (see below). However, preclinical studies suggest that nAChR ligands may hold more promise for the treatment of levodopa-induced dyskinesia (see Table 3). In summary, the effectiveness of long-term treatment with nicotine and various other nAChR agonists has been demonstrated in numerous monkey and rodent studies using oral administration (monkeys) or both oral and various parenteral routes (rodents). Importantly, nAChR agonists can effectively alleviate LID both when given as a pretreatment and when administered after LID has already developed, the latter resembling the more probable clinical use scenario, although some studies do suggest a somewhat delayed onset of alleviation. Equally importantly, antidyskinetic effects by nicotine persist for (at least) several months without tolerance developing. Finally, none of the studies observed any association of a nAChR agonist treatment with a worsening of parkinsonism or a decrease in the antiparkinsonian efficacy of levodopa. This is a particularly significant finding, as nicotine is well-known to modulate striatal dopaminergic function and has also been found to affect levodopa pharmacokinetics in humans (Kyaw et al., 2013).

Studies using selective nAChR agonists and subunit-knockout mice (see Table 3) suggest that the antidyskinetic effects are mediated by several nAChR subtypes. Both β2* nAChR and α7 nAChR agonists can alleviate LID; however, combined treatment did not result in a synergistic effect, suggesting a common final mechanism (Zhang, McGregor, et al., 2014). Mice lacking β2 and α6 subunits developed less severe LID and LID that spontaneously declined over time, respectively, suggesting their involvement in LID pathophysiology (Huang, Grady, and Quik, 2011; Quik et al., 2012). Furthermore, no antidyskinetic effects by nicotine were observed in mice lacking either β2, α6, or α4 nAChR subunits (Huang, Grady, and Quik, 2011; Quik et al., 2012; Quik, Campos, and Grady, 2013). As described above (2.4.2.), nAChRs composed of these subunits (α6β2* and α4(non-α6)β2* nAChRs) are critical regulators of dopaminergic neurotransmission, and thus can be seen as prominent candidates for mediators of the antidyskinetic effects. Although heteromeric

nAChRs on e.g., serotonin terminals (Schwartz et al., 1984; Reuben and Clarke, 2000) could also contribute, findings that nicotine and β2* nAChR agonists are less effective (or even ineffective) in alleviating LID in severely lesioned animals (Huang, Campos, et al., 2011; Huang, Grady, and Quik, 2011; Quik, Campos, and Grady, 2013; Quik, Mallela, Chin, McIntosh, et al., 2013) suggest a major role for nAChRs expressed on dopaminergic neurons. The antidyskinetic effects of α7 agonists, on the other hand, are likely to be mediated by some other nAChR population(s), given that no evidence has been found of α7 expression on striatal dopamine terminals. These could include α7 nAChRs on striatal glutamate terminals (Kaiser and Wonnacott, 2000) or somatodendritic α7 nAChRs on dopaminergic neurons (Klink et al., 2001), although note that the latter may not be expressed in primates (Quik et al., 2000). Also note that in contrast to mice lacking various β2* nAChRs, mice lacking α7 nAChRs developed more severe LID but showed normal sensitivity to alleviation of LID by nicotine (Quik, Campos, and Grady, 2013).

In summary, studies in genetically modified mice suggest that at least α6β2* and α7 nAChRs are involved in LID pathophysiology, possibly exerting opposite effects, while both α4β2* and α6β2* nAChRs mediate the antidyskinetic effects of nicotine. The possible role of α5* nAChRs has not been previously studied. The mechanisms of the LID-alleviating effects remain unclear. However, findings that nicotine and the non-selective nAChR antagonist mecamylamine can have equal and non-additive inhibiting effects on LID suggest receptor blockage as a significant mechanism (Bordia et al., 2010; Bordia, McGregor, McIntosh, et al., 2015). This possibility is supported by findings that chronic nicotine treatment reduced dopamine release from remaining striatal terminals in MPTP-lesioned monkeys (Quik, Chen, et al., 2006) and that antidyskinetic nicotine treatment was associated with reductions in α6β2* nAChR levels and nAChR-mediated dopamine release in rats (Bordia et al., 2013). Such a dampening of potentially excessive striatal dopaminergic activity could occur *via* desensitization and/or downregulation in response to chronic agonist treatment. Supporting desensitization as a potential mechanism is optogenetic evidence suggesting that bursts of large, potentially desensitization-inducing release of acetylcholine from striatal cholinergic interneurons attenuate LID (Bordia et al., 2016). Moreover, when mice with a hypersensitive α6 mutation were treated with nicotine (at a very low but maximum tolerated dose), no LID alleviation was observed; this was suggested to reflect a failure of nicotine to desensitize α6β2* nAChRs (Bordia, McGregor, McIntosh, et al., 2015). Finally, interesting findings suggest that also chronic levodopa treatment itself may result in changes in striatal α3/α6* nAChRs, although changes were only observed in unlesioned monkeys (Quik, Bordia, et al., 2003; Quik et al., 2005).

Extensive preclinical evidence in multiple animal models thus suggests promise for nicotinic receptor ligands as drug treatments for LID. However, it should also be noted that all of the evidence summarized above has been produced by a single research group, with the exception of two monkey studies showing LID alleviation by selective β2* and α7 nAChR agonists (Johnston et al., 2013; Di Paolo et al., 2014). As for clinical evidence, LID

has been associated with increased *post mortem* α7 nAChR binding in the striatum and the GP (Morissette et al., 2016) as well as with increased β2* nAChR density in a brain imaging study (Brumberg et al., 2017), suggesting links between nAChRs and LID in humans as well. Notably, a history of never smoking was also associated with an increased risk of early LID onset in one study (Strong et al., 2006). However, published clinical trials of nicotinic agonists have not so far demonstrated antidyskinetic effects (see below).

Clinical studies on nicotinic receptor agonists in Parkinson's disease

To date, a number of clinical studies have investigated the safety and efficacy of nAChR agonists in the treatment of the symptoms of PD (see Table 4). In summary, although nAChR agonists are usually acceptably tolerated and show promising effects in uncontrolled studies, beneficial clinical effects on motor or non-motor symptoms of PD have not been demonstrated in well-controlled trials. Potential shortfalls such as a too short treatment time or a too low dosage appear to have been ruled out (Villafane et al., 2018). Significant placebo effects may be involved, particularly given benefits in unblinded but not blinded measures in one study (Villafane et al., 2018). Note that all of the controlled studies on the effects of nicotine included only non-smoking patients. Publication of results from a few recent controlled clinical studies is awaited at the time of writing. According to published conference abstracts, preliminary results of one study in 65 PD patients suggest reductions in falls, freezing of gait, and possibly LID after 10 weeks of administration of an oral nicotine formulation (Lieberman et al., 2018), while in another study in 163 early PD patients one year of transdermal nicotine administration failed to improve motor symptoms (Oertel et al., 2018).

The lack of efficacy against parkinsonian motor symptoms is in fact in line with most preclinical results (see above). However, it remains an open question whether nAChR agonists might have beneficial clinical effects on some specific motor or non-motor symptoms or, in particular, motor fluctuations such as LID. Measures of LID have so far been reported in only two published studies. A reduction in troublesome LID by nicotine was observed as an unblinded outcome (Villafane et al., 2018), while an α7 agonist previously effective in a monkey model had no effect on LID (Trenkwalder et al., 2016). It also remains possible that other selective nAChR agonists than those studied so far might show clinical benefit. As for neuroprotective effects by nAChR agonists, while preclinical studies strongly suggest potential benefits, neuroprotection specifically has not been studied clinically. Indeed, any neuroprotective effects might be difficult to observe in diagnosed PD patients, given the significant nigrostriatal damage already at the time of symptom appearance (Fearnley and Lees, 1991) as well as a lack of validated biomarkers (AlDakheel et al., 2014). Some preclinical studies also suggest significant neuroprotective effects by nicotine only when the treatment is initiated before the loss of dopaminergic neurons (Huang et al., 2009). Here, the question then becomes a need for earlier diagnosis of PD or identification of increased disease risk, and the feasibility and safety of pre-treatment with nAChR agonists.

Table 3. Studies on antidyskinetic effects of nAChR ligands in monkey, rat, and mouse models. Post-treatment = treatment started after maximal LID had already developed. LD = levodopa; WT = wild-type; KO = knock-out; [1]Positive nicotine control results omitted

Study	Model	Drug treatment(s)	Main treatment outcomes
Quik et al., 2007	Monkey, MPTP	Nicotine, drinking water, pretreatment for 8–9 weeks	Reduced LID in both naïve and LD-primed animals, persisted for 8 weeks
Quik, Mallela, Chin, McIntosh, et al., 2013	Monkey, MPTP	Nicotine, drinking water, pretreatment for 9 weeks	Reduced LID in moderately but not severely lesioned animals
Quik, Mallela, Ly, and Zhang, 2013	Monkey, MPTP	Nicotine, drinking water, pretreatment for 8 weeks pretreatment for 2 weeks post-treatment 8 or 4 weeks after LD	Reduced LID, fast onset (2 weeks) Reduced LID, delayed (8 w), persisted 23 w Reduced LID, mixed onset (5 or 11 weeks)
Zhang et al., 2013[1]	Monkey, MPTP	Varenicline, oral, escalating doses, 2/d for 11 weeks, with LD β2* agonist, oral, escalating doses, 2/d for 7 weeks, with LD	Reduced LID Reduced LID
Zhang, Bordia, et al., 2014[1]	Monkey, MPTP	β2* agonists, oral, escalating doses, 2/d for 5–12 weeks, with LD	Reduced LID
Zhang, McGregor, et al,. 2014[1]	Monkey, MPTP	α7 and/or β2* agonist, oral, various regimes, with LD	Reduced LID, no synergistic effect
Zhang et al., 2015	Monkey, MPTP	Nicotine, drinking water, moderate lesion – pretreatment for 1 wk Nicotine, drinking water, severe lesion – post-treatment 5 wks after LD α7 agonist, oral, various regimes, with LD, moderate vs. severe lesion β2* agonist, oral, various regimes, with LD, moderate vs. severe lesion	Reduced LID, fast onset (1 week) Reduced LID, fast onset (3 weeks) Reduced LID, delayed in severe lesion Reduced LID only in moderate lesion
Johnston et al., 2013	Monkey, MPTP	β2* agonist, oral, 3 doses, 2/d for 2 wks each, post-treatment	Reduced LID
Di Paolo et al., 2014	Monkey, MPTP	α7 agonist, nasogastric gavage, 3 doses acutely, post-treatment	Reduced LID
Bordia et al., 2008	Rat, 6-OHDA	Nicotine, drinking water and minipump, pretreatment and post-treatment	Reduced LID, persisted for 15 weeks

Table 3 cont.

Study	Model	Drug treatment(s)	Main treatment outcomes
Bordia et al., 2010	Rat, 6-OHDA	Nicotine or nAChR antagonist, injection, 1 x, post-treatment Nicotine, injection, 1–2/d for 4–10 d or minipump, post-treatment nAChR antagonist, injection, 1/d for 4 d, post-treatment	No effect Reduced LID Reduced LID, no synergism with nicotine
Huang, Campos, et al., 2011	Rat, 6-OHDA	Varenicline, injection, 2/d for 4d, post-treatment, partial / near-total lesion β2* agonist, similar administration	Reduced LID only in partial lesion Reduced LID more in partial lesion
Bordia et al., 2013	Rat, 6-OHDA	Nicotine, minipump, pretreatment	Reduced LID, persisted for 12 weeks
Quik, Campos, Bordia, et al., 2013	Rat, 6-OHDA	Various β2* agonists, minipump, pretreatment, near-total lesion	Reduced LID, persisted for 11 weeks
Huang, Grady, and Quik, 2011	Mouse, 6-OHDA	Nicotine, drinking water, pretreatment and post-treatment Nicotine, drinking water, post-treatment, WT + β2-KO	Reduced LID, persisted for 15 weeks β2-KO: Less severe LID, no effect by nicotine
Quik et al., 2012	Mouse, 6-OHDA	Nicotine, drinking water, post-treatment, WT + α6-KO	α6-KO: Less severe LID, no effect by nicotine
Quik, Campos, and Grady, 2013	Mouse, 6-OHDA	Nicotine, drinking water, post-treatment, WT + α4-KO Nicotine, drinking water, post-treatment, WT + α7-KO	α4-KO: No effect by nicotine α7-KO: More severe LID, nicotine reduced LID
Bordia, McGregor, McIntosh, et al., 2015	Mouse, 6-OHDA	Nicotine, drinking water, post-treatment, hypersensitive α6 nAChR antagonist, injection, 1 x acutely, WT + hypersensitive α6	No effect by maximal tolerated dose Reduced LID in both genotypes

Table 4. Clinical studies on the effects of nicotinic receptor agonists in Parkinson's disease patients.

Study	Treatment	Design	Patients (N)	Outcome
Moll, 1926	Nicotine injection, acute	Case reports	Postencephalitic parkinsonism (13)	Reduced rigidity in 9 cases. Persisted up to 6 months. Many acute adverse effects.
Ishikawa and Miyateke, 1993	Smoking, nicotine gum, acute	Case reports	Early-onset PD (6), late-onset PD (52)	Improvement in tremor and akinesia lasting 5–30 min, only in early-onset PD. Lesser effect with gum.
Fagerström et al., 1994	Nicotine gum and patch, long-term	Crossover case reports	Moderate PD (2)	1: Persisting improvement in tremor and rigidity 2: Persisting improvement in mood and function
Kelton et al., 2000	Nicotine patch (up to 14 mg/day), 2 weeks	Open-label, no placebo	Moderate PD (15)	Improvements in motor and cognitive performance. Well-tolerated.
Villafane et al., 2007	Nicotine patch (up to 105 mg/day), 17 weeks	Open-label, no placebo	Advanced PD (6)	Improvement in motor symptom scores, reduced dopaminergic treatment. Acceptably tolerated.
Clemens et al., 1995	Nicotine gum (2 mg), acute	Placebo-controlled, double-blind	Moderate PD (48)	No effect on motor symptom scores. Acceptably tolerated.
Ebersbach et al., 1999	Nicotine patch (14 mg/day), 12 h	Placebo-controlled, double-blind	Moderate PD (16)	Worsening in motor symptom scores. Well-tolerated.
Vieregge et al., 2001	Nicotine patch (up to 14 mg/day), 3 weeks	Placebo-controlled, double-blind	Moderate PD (32)	No effect on motor performance or motor symptom scores. Well-tolerated.
Lemay et al., 2004	Nicotine patch (up to 21 mg/day), 25 days	Controlled open-label, no placebo	Moderate PD (22)	No effect on motor or cognitive performance compared to matched control subjects. Poorly tolerated.
Villafane et al., 2018	Nicotine patch (up to 90 mg/day), 39 weeks	Open-label, blinded-endpoint, no placebo	Moderate to severe PD (40)	No effect on motor symptom scores (blinded). Improvements in unblinded measures (levodopa dosage, motor fluctuations). Acceptably tolerated.
The Parkinson Study Group, 2006	α4β2* agonist, 4 weeks	Placebo-controlled, double-blind	Moderate PD (77)	No effect on motor symptom scores or cognitive tests. Poorly tolerated at higher dosages.
Trenkwalder et al., 2016	α7 agonist, 28 days	Placebo-controlled, double-blind	Moderate to severe PD with LID (71)	No effect on motor symptom or LID scores. Well-tolerated.

3. Aims of the studies

As discussed above, the current treatment options for Parkinson's disease and treatment-related adverse effects such as levodopa-induced dyskinesia remain suboptimal. The studies comprising this thesis aimed at addressing the need for novel treatment options by investigating the role of neuronal nicotinic acetylcholine receptors in Parkinson's disease and levodopa-induced dyskinesia and their potential usability as drug targets. More specifically, the aims of the present studies were:

1. To study for the first time the role of the α5* nicotinic acetylcholine receptor subtype in mouse models of Parkinson's disease and levodopa-induced dyskinesia (Study I).

2. To study the effectiveness of nicotinic receptor agonists in alleviating levodopa-induced dyskinesia in mouse models of moderate and advanced Parkinson's disease (Studies II–III).

3. To study the mechanisms of antidyskinetic nicotine treatment by investigating the effects of chronic nicotine administration on striatal dopamine release (Study II).

4. To study the links between the brain-derived neurotrophic factor, levodopa-induced dyskinesia, and the antidyskinetic effects of nicotine (Study III).

5. To improve the methods of stereotactic surgery and postoperative care relating to near-total unilateral dopaminergic denervation induced by intra-MFB 6-OHDA administration in mice (Study IV).

4. Materials and methods

Table 5 lists all experimental methods used in the studies and the author's personal contribution.

Table 5. Methods used in each of the original studies and the author's personal contribution. Major contribution = significant contribution to study design and data analysis along with significant contribution to hands-on work and/or direct supervision of hands-on work; Minor contribution = significant contribution to study design and data analysis and/or minor contribution to hands-on work

Method	Original studies	Author's personal contribution	
		Major	Minor
BDNF ELISA	III		
Densitometric analyses	I, II, III	X	
Dopamine HPLC	I		X
Dopamine receptor qPCR	I		
Dopamine release assays	II	X	
Dopamine transporter Western blotting	I	X	
Dopamine uptake assays	I	X	
Drug-induced locomotor activity	I	X	
Drug-induced rotametry	I		X
Immunohistochemistry	I, II, III	X	
Mouse model of levodopa-induced dyskinesia	I, II, III	X	
Mouse strain maintenance and genotyping	I	X	
Stereological cell counting	I	X	
Stereotactic surgeries and postoperative care	I, II, III, IV		X

4.1. Drugs

6-hydroxydopamine HCl, amantadine HCl, apomorphine HCl, benserazide HCl, desipramine HCl, dopamine HCl, levodopa methyl ester HCl, (–)-nicotine, nicotine hydrogen tartrate, nomifensine maleate and pargyline HCl were from Sigma-Aldrich (St. Louis, MO, USA). [^{3}H]dopamine was from PerkinElmer (Waltham, MA, USA). α-conotoxin MII was a gift from Dr. J. M. McIntosh (University of Utah, UT, USA). AZD0328 ((2´R)-spiro-[1-azabicyclo[2.2.2]octane-3,2´(3´H)-furo[2,3-b]pyridine] D-tartrate) was provided free of

charge by AstraZeneca (Cambridge, MA, USA). Isoflurane was from Piramal Healthcare (Morpeth, UK). Lidocaine and sodium pentobarbital solutions were from Orion Pharma (Espoo, Finland), buprenorphine solution was from RB Pharmaceuticals (Berkshire, UK), and carprofen solution was from Pfizer Animal Health (Helsinki, Finland). D-amphetamine sulphate was synthesized at the Faculty of Pharmacy (University of Helsinki, Finland). Doses of drugs refer to free bases. Drugs were administered *in vivo* in saline at a volume of 10 ml/kg.

4.2. Animals

α5-knockout (α5-KO) C57BL/6J (Salas et al., 2003) and wild-type (WT) mice used in Study I were obtained from the Institute for Behavioral Genetics, University of Colorado (Boulder, CO, USA), bred at the research site, and genotyped as described by Salminen et al. (2004). C57BL/6JRccHsd mice (Envigo, Horst, Netherlands) were used in all other studies. See Figure 10 for the age of animals in each experiment. Experimental groups that were directly compared were carefully balanced with respect to mean age unless otherwise stated. The sex distribution in each experiment is given in the Results section. Most studies using 6-OHDA injections into the medial forebrain bundle (MFB) included only females due to penile prolapse complications after a severe lesion (Thiele et al., 2011). Animals were group housed in a temperature- and humidity-controlled environment under a 12 h light/dark cycle. All experiments were authorized by the national Animal Experiment Board of Finland (permit number ESAVI/198/04.10.07/2014). In some experiments, the mice were habituated to handling prior to any procedures using a progressive handling protocol (see Study IV).

4.3. Stereotactic 6-OHDA administration and postoperative care

Unilateral lesioning of the nigrostriatal pathway was induced by stereotactic injections of 6-OHDA under isoflurane anesthesia. To model moderate and advanced Parkinson's disease, two 6-OHDA models with different sites of injection (striatum and MFB, respectively) were used. 6-OHDA was dissolved in 0.02 % ascorbate-saline and injected using a stereotaxic frame (Stoelting, Wood Dale, IL, USA) at the coordinates given below, relative to the bregma and the dural surface. In the intrastriatal model, two 1 µl injections containing 6 µg 6-OHDA each were administered into the left dorsal striatum. In Study I, the sites were 1: A/P +1.0; L/M +1.9; D/V −2.9, and 2: A/P +0.3; L/M +2.0; D/V −2.9. In Study II, the sites were identical except for being slightly more lateral: 1: L/M +2.1; 2: L/M +2.3. In the intra-MFB model, one 0.2 µl injection containing 3 µg 6-OHDA was administered into the right MFB at A/P -1.2; L/M -1.1; D/V −5.0. In Studies I and II, desipramine (25 mg/kg, i.p.) was administered 30 min prior to surgery to inhibit noradrenergic neurodegeneration. Lidocaine, buprenorphine (0.1 mg/kg, s.c.), and carprofen (5 mg/kg, s.c.) were used for pain relief.

The postoperative care necessary to ensure the survival of operated hemiparkinsonian mice was improved throughout the studies, and in its final form was provided for 14 consequtive days and included daily systematic welfare assessment, warm saline injections to prevent dehydration, food pellets softened by soaking, additional high-energy diet (Bacon Softies, Bio-Serv, Flemington, NJ, USA; Nutriplus gel, Virbac, Carros, France), feeding by hand, and alleviating hypothermia with heating pads. See Study IV for more details on the postoperative care. Postoperative mortality, mostly due to euthanasia, was (listed in chronological order) 11 out of 39 (28 %) in Study II (intrastriatal model), 32 out of 56 (57 %) in Study I pilot experiments (intra-MFB model), 1 out of 15 (7 %) in Study III (intra-MFB), and 2 out of 22 (9 %) and 3 out of 22 (14 %) in Study I main experiments (intrastriatal and intra-MFB, respectively).

4.4. *In vivo* experiments

4.4.1. Drug-induced rotametry and locomotor activity (Study I)

Rotation induced by dopaminergic drugs, reflecting the interhemispheric dopaminergic imbalance in unilateral parkinsonism, was measured 2–3 weeks after the 6-OHDA injections. A Roto-Rat automated rotametry apparatus (Med Associates, St. Albans, VA, USA) was used. Mice were administered amphetamine (2.5 mg/kg, i.p.) or apomorphine (0.5 mg/kg, i.p.), attached to automatic detectors with cable ties and an iron wire, and placed in a plexiglass cylinder (11 x 15 cm). Rotations were measured at 5 min intervals and expressed as net ipsi- or contralateral rotations.

The effect of amphetamine on locomotion in intact mice was measured using an automated infrared activity monitor (Activity Monitor, Med Associates). Mice were placed in a 43 x 43 cm plexiglass chamber for 30 min, after which amphetamine (2.5 mg/kg, i.p.) was administered. The distance travelled by the animal was measured at 5 min intervals *via* photobeam interruption.

4.4.2. Drug treatments

To induce dyskinesia, lesioned mice were administered levodopa and the AADC inhibitor benserazide daily (Mon-Fri) in a single s.c. injection. Dosages were chosen on the basis of literature (Lundblad et al., 2004; Francardo et al., 2011; Thiele et al., 2011). Note that two different strategies of levodopa:benserazide dosing were used, where in later studies the benserazide dose was lowered to match the 4:1 ratio used clinically (Thiele et al., 2011). In Study II, nicotine treatment was initiated simultaneously with the levodopa treatment. In all other experiments, other drug treatments were initiated after LID had already developed to better mimic the potential clinical use scenario.

See Figure 10 for the time courses of the drug treatments in each experiment. In Studies I–III, nicotine was administered in pH-adjusted drinking water with no other source of

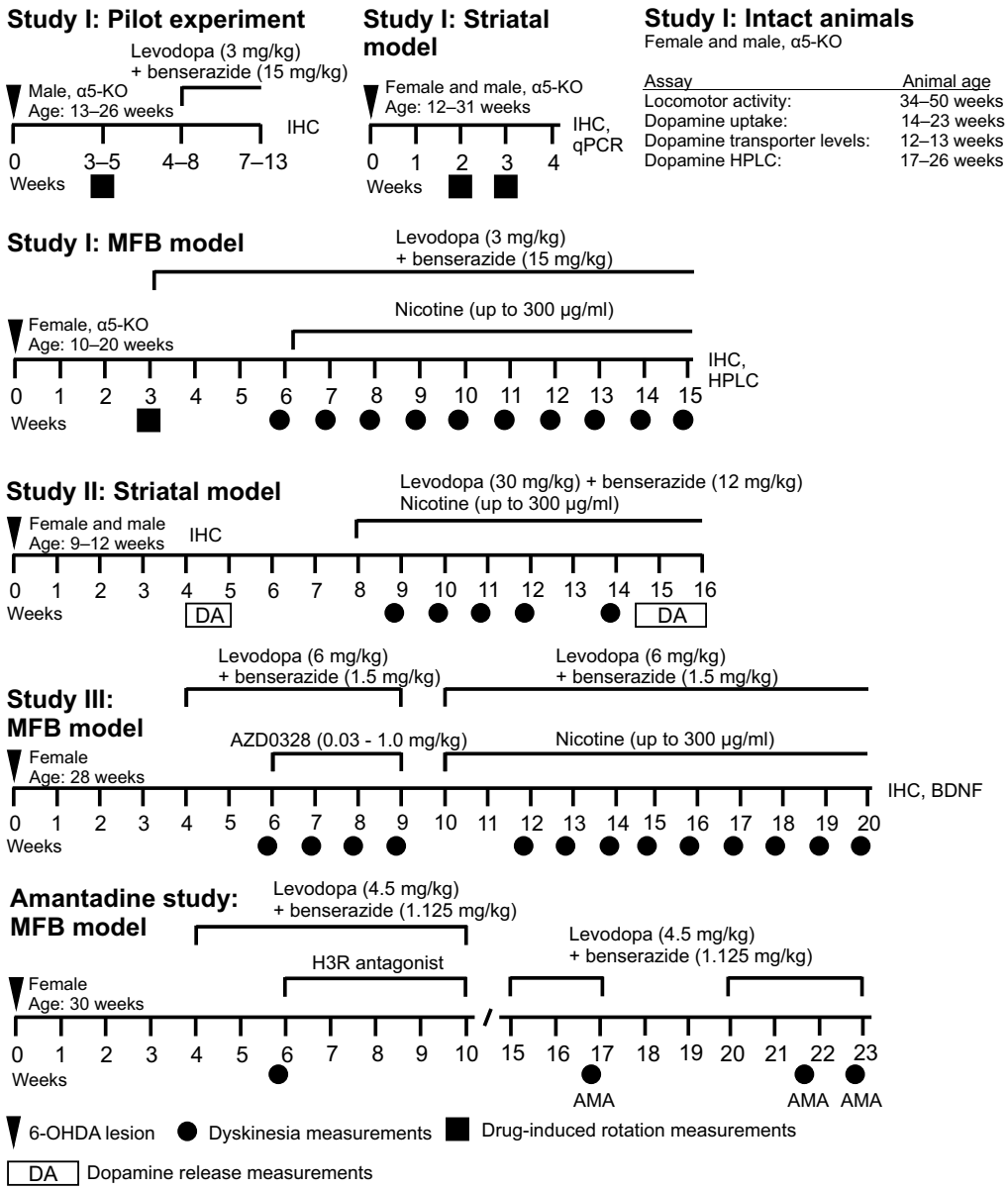

Figure 10. Summary of the experiments discussed within this thesis. Shown are time course, lesion model, sex and age at the time of lesioning, drug treatments, and *in vivo* and *ex vivo* measurements performed. α5-KO refers to experiments including mice lacking the α5 nicotinic receptor subunit. Study order does not reflect the chronological order of the experiments. 6-OHDA = 6-hydroxydopamine; AMA = amantadine; BDNF = brain-derived neurotrophic factor; HPLC = high-performance liquid chromatography; IHC = immunohistochemistry; MFB = medial forebrain bundle; qPCR = quantitative polymerase chain reaction

fluid available. The nicotine concentration was gradually raised to 300 µg/ml during 2–3 weeks as previously described (Pekonen et al., 1993; Pietilä et al., 1995). In Studies I and III, saccharin (2 %) was added to both nicotine and control solutions to mask the taste of nicotine (Huang, Grady, and Quik, 2011). Water consumption was measured every 2–3 days. In Study III, the α7 nAChR partial agonist AZD0328 (Sydserff et al., 2009) was administered for three weeks at escalating doses of 0.03 mg/kg, 0.1 mg/kg, and 1.0 mg/kg (s.c.). Each dose was given for five days 30 min prior to levodopa administration, with control mice receiving saline injections, and dyskinesia severity was assessed on the fifth day. Amantadine was administered acutely in an additional series of experiments, following chronic administration of a histamine H3 receptor antagonist (not discussed in this thesis) and a prolonged washout. First, 60 mg/kg amantadine (i.p.) or saline was administered 60 min before levodopa. Following a second washout, 60 mg/kg amantadine or saline was administered 0 or 100 min before levodopa. Finally, a week later 5 or 15 mg/kg amantadine or saline was administered 60 min before levodopa.

4.4.3. Assessment of dyskinesia severity

Following the levodopa injection, mice were individually video recorded in transparent cylinders flanked by two vertical mirrors using a web camera (HD Pro C920, Logitech, Newark, CA, USA). In Study II, mice were recorded every 30 min for 60 s and remained in the cylinder throughout. In all other studies, mice were recorded every 20 min for 60 s and were returned to the home cage in between. Dyskinesia severity was assessed from the recordings by a researcher blinded to the experimental group and the time after levodopa injection. Dyskinesia were classified into three subtypes (axial, orolingual, and forelimb dyskinesia) based on topographic distribution and rated on a scale of 0–4 according to criteria assessing both amplitude and frequency of dyskinetic behaviors (Table 6). The scoring criteria were developed on the basis of previously published scoring methods (Lundblad et al., 2004; Cenci and Lundblad, 2007; Thiele et al., 2011) and the video recordings of LID of varying severity obtained in the present studies. See 6.1.2. for discussion on the novel scoring method. Some recordings were additionally scored using the method of Lundblad et al. (2004). Mice that did not develop dyskinesia (16 % of all levodopa-treated mice) were not included in any dataset.

Scores of each dyskinesia subtype (0–4) as well as total dyskinesia scores (sum of the subtype scores, 0–12; Lundblad et al., 2004) were analyzed. Integrated weekly scores were calculated as the sum or the area under the curve of the scores obtained within one recording session (Cenci and Lundblad, 2007). Finally, integrated dyskinesia scores across multi-week experiments were calculated as the area under the curve of the weekly scores.

Table 6. Criteria used for scoring dyskinetic behavior in mice. For axial dyskinesia, the given behavior had to occur more than once per minute. Half points could be given when considered appropriate.

Subtype	Score	Description
Axial dyskinesia	0	Normal physiological motor repertoire.
	1	Contralateral deviation of the head and trunk with one or both forelimbs contacting the ground. Mouse is steady or moving.
	2	Contralateral deviation of the head and trunk in a bipedal sitting position.
	3	Contralateral deviation of the head and trunk in a bipedal sitting position, causing **loss of balance**.
	4	In a bipedal sitting position, head and trunk **fixed and in a severely twisted position** followed by a loss of balance.
Orolingual dyskinesia	0	Normal physiological motor repertoire.
	1	Vacuous chewing movements OR **occasional** (≤10 times per minute) biting of the contralateral side/limbs.
	2	Vacuous chewing movements, including tongue protrusions OR **repeated** (11–19 times) biting of the contralateral side/limbs.
	3	**Sustained** (3–5 s) vacuous chewing movements, including tongue protrusions, OR **frequent** (≥20 times) or **sustained** (3–5 s) biting of the contralateral side/limbs.
	4	**Prolonged** (>5 s) vacuous chewing movements, including tongue protrusions, OR **prolonged** (>5 s) biting of the contralateral side/limbs.
Forelimb dyskinesia	0	Normal physiological motor repertoire.
	1	Isolated jerky movements of the contralateral distal forelimb.
	2	Repetitive, **small** movements (vertical or horizontal) of the contralateral forelimb involving the distal and proximal forelimb; <50 % of time.
	3	Repetitive, small movements of the contralateral forelimb as in 2; 50–100 % of time.
	4	Repetitive, **large** movements (vertical or horizontal) of the contralateral forelimb involving the distal and proximal forelimb.

4.5. Immunohistochemistry

4.5.1. Tyrosine hydroxylase immunostaining

Dopaminergic denervation was quantified from brain sections stained for the dopaminergic neuron-specific enzyme TH. Mice were either anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused intracardially with phosphate-buffered saline (PBS) followed by 4 % paraformaldehyde (PFA) in PBS, pH 7.4 (Study I pilot, Study II); or killed by cervical dislocation with the posterior part of the brain containing the SN dissected on ice. In both cases, the brains were immersed overnight in 4 % PFA in PBS at +4 °C and then stored in 20 % sucrose in PBS at +4 °C. Subsequently, the brains were frozen in isopentane on dry ice and stored at -80 °C. Free-floating coronal sections were cut with a Leica CM3050 cryostat (Leica Biosystems, Nussloch, Germany) and stored at -20 °C. Sections were cut at 40 µm (Study I, intrastriatal model) or 30 µm thickness.

TH immunostaining followed a previously described protocol (for a detailed description, see Mijatovic et al., 2007) with minor variations. In brief, sections were washed in PBS and blocked first by H_2O_2 and then by normal goat serum. Next, the sections were incubated overnight at +4 °C with the primary antibody (rabbit anti-TH, 1:2000; AB152, Millipore, Bedford, MA, USA). After washing in PBS, the sections were incubated for 1 h with either the secondary antibody (biotinylated goat anti-rabbit, 1:500; BA1000, Vector Laboratories, Burlingame, CA, USA) or biotinylated protein A (see below). Subsequently, the sections were washed in PBS, and the staining was reinforced using the avidin-biotin complex method (Vectastain Elite ABC kit, Vector Laboratories) and visualized with diaminobenzidine. The stained sections were dehydrated in graded ethanols, cleared in xylene, and coverslipped with Depex mounting medium (VWR International, Poole, UK).

The use of biotinylated protein A was an exception to the previously described protocol. The reagent was prepared using protein A (MP Biomedicals, Santa Ana, CA, USA) and N-hydroxysuccinimido-biotin (Sigma-Aldrich). In brief, protein A was incubated for 1 h with N-hydroxysuccinimido-biotin, and the reaction mixture dialyzed overnight at +4 °C (membrane cutoff 12–14 kDa, SpectraPor 4, Spectrum Laboratories, Rancho Dominguez, CA, USA). The mixture was then stored at -20 °C, and used as 1:100 in PBS with 0.5 % bovine serum albumin.

4.5.2. Densitometric and stereological analyses

Both densitometric quantification of immunostaining and stereological counting of stained cell bodies were used. For densitometric analyses, stained brain sections were imaged using a 3DHISTECH slide scanner (3DHISTECH, Budapest, Hungary). Optical density across the SNC (Fu et al., 2012) and/or the dorsal striatum (Franklin and Paxinos, 1997) was measured using ImageJ 1.6 (NIH, Bethesda, MD, USA). Optical density of background staining was subtracted and the results expressed as percent of intact side. The lesion extent

in the SNC and the striatum was calculated as the mean of three consecutive sections (every 6th section) between −2.9 and −3.4 mm or +0.1 and +1.0 mm from the bregma, respectively.

In Study I, the numbers of TH-positive neurons in the dorsal tier (SNCD) and the medial cluster (SNCM) of the SNC were estimated by blinded unbiased stereological cell counting. Demarcation of brain areas followed published delineations (Franklin and Paxinos, 1997; Fu et al., 2012). Three consecutive sections (every third section in the striatal model, every sixth section in the MFB model) were selected between levels −2.9 and −3.4 mm or between −3.1 and −3.6 mm from the bregma for the SNCD and the SNCM, respectively. StereoInvestigator (MBF Bioscience, Williston, VT, USA) was used to first outline the region at 4x magnification and then count stained cell bodies with an optical fractionator, according to optical disector rules (Gundersen et al., 1988), at regular intervals (SNCD: x = 80 mm, y = 80 mm; SNCM: x = 60 mm, y = 60 mm) within a counting frame (60 mm x 60 mm) superimposed on an image obtained using a 60x oil objective (Olympus Plan/Apo, Olympus, Tokyo, Japan). Gundersen's coefficients of error (CE) were ≤0.15 for the intact hemisphere. Data were expressed as percentage of the intact hemisphere.

4.6. *Ex vivo* assays

4.6.1. Synaptosomal [^{3}H]dopamine uptake (Study I)

To study the effects of the lack of α5* nAChRs on DAT function, dopamine uptake into synaptosomes was measured using tritiated dopamine as a marker. Intact mice were killed by cervical dislocation and samples of the striatum collected on ice using tweezers. The samples were homogenized by hand using a glass-teflon homogenizer in 2 ml of ice-cold homogenization buffer (0.32 M sucrose, 5 mM HEPES, pH 7.5). A 200 µl aliquot was centrifuged at 1000 g for 10 min at +4 °C followed by centrifugation of the first supernatant at 12000 g for 20 min at +4 °C. The pellet was resuspended in 800 µl of uptake buffer (128 mM NaCl, 2.4 mM KCl, 3.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10 mM glucose, 1 mM ascorbic acid, 10 µM pargyline, 25 mM HEPES pH 7.5, 0.1 % bovine serum albumin). The uptake assay was then performed in a MultiScreen HTS 96-well filter-bottomed plate (Millipore, Bedford, MA, USA) in a volume of 100 µl uptake buffer containing 25 µl of the synaptosome suspension and 1 µM dopamine (2 % [^{3}H]dopamine). 200 µM nomifensine was used for blank determination. Solutions were incubated for 30 min at room temperature before aspiration and washing the filters with 6 x 200 µl cold uptake buffer. OptiPhase Supermix scintillation cocktail (100 µl/well; PerkinElmer) was added, and radioactivity measured with liquid scintillation counting (5 min per well; 1450 MicroBeta TriLux; Wallac, Turku, Finland). The protein concentrations of synaptosomal suspensions were measured using the Bradford method (Bradford Reagent, Sigma-Aldrich). Data were expressed as pmol of dopamine taken up per µg of protein.

4.6.2. Dopamine transporter Western blotting (Study I)

To study the effects of the lack of α5* nAChRs on DAT expression, DAT, phospho (T53)-DAT (pDAT), and β-actin protein levels were measured using the methods of Julku et al. (2018) with some modifications. Intact mice were killed by cervical dislocation, and the brains were rapidly dissected, frozen in isopentane on dry ice, and stored at -80 °C. Tissue samples from the dorsal striatum were collected in a Leica CM3050 cryostat using a sample corer (for a detailed description, see Julku et al., 2018). Samples were homogenized using ultrasound (GM35-400, Rinco Ultrasonic, Romanshorn, Switzerland) in five volumes of ice-cold modified RIPA buffer (50 mM Tris pH 7.4, 1 % NP-40, 0.25 % sodium deoxycholate, 150 mM NaCl) additionally containing Halt Phosphatase and Protease Inhibitor cocktails (Thermo Fisher Scientific, Rockford, IL, USA). The homogenates were centrifuged at 16000 g for 15 min at +4 °C and the supernatants collected. Protein concentrations were measured with the BCA method (Pierce BCA Assay Kit, Thermo Fisher Scientific).

Samples were diluted in Laemmli buffer (Bio-Rad, Hercules, CA, USA) containing β-mercaptoethanol and boiled for 5 min, after which 30 µg of protein was loaded onto 4–20 % (DAT) or 8–16 % (pDAT) Mini-PROTEAN TGX precast gels (Bio-Rad). Gels were ran for 30 min at 200 V in running buffer (25 mM Tris, 190 mM glycine, 0.1 % sodium dodecyl sulphate) and transferred to Trans-blot Turbo midi nitrocellulose membranes (Bio-Rad) using a Trans-blot Turbo Transfer System (Bio-Rad). Membranes were blocked for 1 h at room temperature in 5 % skim milk in 0.05 % Tween-20 in Tris-buffered saline (TTBS), incubated with the primary antibody diluted in 5 % skim milk in TTBS overnight at +4 °C, washed in TTBS, and incubated with the secondary antibody diluted in 5 % skim milk in TTBS for 2 h at room temperature. The following primary antibodies were used: DAT, rabbit anti-DAT (1:1000, #PA1-4656, Thermo Fisher Scientific); pDAT, rabbit anti-phospho(T53)-DAT (1:500, #ab183486, AbCam, Cambridge, UK); β-actin, rabbit anti-β-actin (1:2000, #ab8227, AbCam). The secondary antibody used was goat anti-rabbit horseradish peroxidase (1:2000, #31460, Thermo Fisher Scientific). After washing the membranes in TTBS and in TBS, they were incubated for 5 min with SuperSignal West Pico (DAT, β-actin) or Femto (pDAT) chemiluminescence substrate (Thermo Fisher Scientific Scientific). Images were captured with a LI-COR C-digit chemiluminescence scanner (LI-COR, Lincoln, NE, USA). Optical density values were measured using ImageJ 1.6 and normalized to loading control (β-actin) optical density values, with the data expressed as percentage of wild-type mean.

4.6.3. Dopamine and metabolite HPLC (Study I)

The effects of the lack of α5* nAChRs on striatal tissue levels of dopamine and its metabolites were studied using high-performance liquid chromatography (HPLC). Intact mice were killed by cervical dislocation, and samples of the dorsal striatum were collected using a chilled mouse brain matrix and a sample corer (for a detailed description, see Airavaara et al., 2004) and stored at -80 °C. Tissue concentrations of dopamine,

3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were measured using methods described by Julku et al. (2018). In brief, the samples were homogenized using ultrasound (GM35-400, Rinco Ultrasonic) in 0.5 ml of homogenization solution consisting of six parts of 0.2 M $HClO_4$ and one part of antioxidant solution (oxalic acid, acetic acid, L-cysteine). The homogenates were centrifuged at 20800 g for 35 min at +4 °C. Supernatants were transferred to 0.5 ml Vivaspin filter concentrators (10000 MWCO PES; Vivascience, Hannover, Germany) and centrifuged at 8600 g for 35 min at +4 °C. The filtrates were then analyzed with HPLC with electrochemical detection. See Julku et al. (2018) for a detailed description of the HPLC system. Chromatograms were processed and analyte concentrations calculated using CoulArray (ESA Biosciences, Chelmsford, MA, USA). Data were expressed as µg of analyte per gram of wet tissue.

4.6.4. Dopamine receptor qPCR (Study I)

The effects of the 6-OHDA lesion and the lack of α5* nAChRs on dopamine receptor expression were studied by measuring D1R and D2R mRNA with the quantitative polymerase chain reaction (qPCR) method. Mice lesioned with intrastriatal 6-OHDA were killed by cervical dislocation, and samples of the dorsal striatum of both hemispheres were collected using a chilled mouse brain matrix and a sample corer (Airavaara et al., 2004) and stored at -80 °C. Tissue from age- and sex-matched control mice (C57BL/6JRccHsd) was pooled in two groups to have separate native controls for both hemispheres. RNA was isolated using an RNAeasy Mini kit (Qiagen, Hilden, Germany) and DNase digestion performed using an RNase-Free Dnase set (Qiagen) as described by the manufacturer. RNA was quantified with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and converted to cDNA by Super-Script III First-Strand Synthesis SuperMix and oligo(dT) primers (Invitrogen, Carlsbad, CA, USA). For qPCR, cDNA was diluted with RNase free water, and 4.5 ml of cDNA used per well in a 10 ml reaction volume. ABsolute Blue QPCR Mix, ROX (Thermo Scientific, Waltham, MA, USA) was mixed with TaqMan primers and hydrolysis probes (Applied Biosystems, Foster City, CA, USA; catalogue numbers: Dopamine 1 receptor, Mm02620146_s1; Dopamine 2 receptor, Mm00438545_m1). PCR amplification was performed on 384-well plates using a Roche LightCycler (Roche Diagnostics, Mannheim, Germany) with 1 cycle of 15 min at 95 °C, 40 cycles of 15 s at 95 °C, and 40 cycles of 1 min at 60 °C by turns. Expression of target genes was normalized to the pooled control with the reference gene GAPDH (TaqMan, Mm99999915_g1). Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method and data expressed as fold change in mRNA levels (Livak and Schmittgen, 2001).

4.6.5. Synaptosomal [^{3}H]dopamine release (Study II)

To study the effects of the 6-OHDA lesion and chronic nicotine treatment on striatal dopamine release, the release of dopamine from synaptosomes was measured using tritiated dopamine as a marker. Mice lesioned with intrastriatal 6-OHDA were assayed either after the lesioning or at the end of chronic drug treatments (see Figure 10). Mice were killed by

cervical dislocation 1 h after the last levodopa administration, and samples of the dorsal and ventral striatum collected using a chilled mouse brain matrix and a sample corer (for detailed description see Airavaara et al., 2004). Subsequently, the methods of Salminen et al. (2004) were followed with minor modifications. The samples were homogenized by hand using a glass-teflon homogenizer in 500 µl of ice-cold homogenization buffer (see 4.6.1.). The homogenate was combined with two 500 µl rinses of the homogenizer and centrifuged at 12000 g for 20 min at +4 °C. The pellet was resuspended in 800 µl of uptake buffer (see 4.6.1., not containing bovine serum albumin) and incubated for 10 min at +37 °C. Then, 4 µCi of [^{3}H]dopamine (final concentration ~0.1 µM) was added, and the suspension incubated for 5 min at +37 °C. Aliquots of 80 µl were distributed to parallel filters and superfused at room temperature with superfusion buffer (uptake buffer additionally containing 1 µM nomifensine, 1 µM atropine and 0.1 % bovine serum albumin) at ~0.8 ml/min. After 10 min of superfusion to wash off [^{3}H]dopamine not taken up, the synaptosomes were stimulated by switching for 20 s to superfusion buffer containing 10 µM nicotine. To determine α4β2*-mediated release, 50 nM α-conotoxin MII was added to the superfusion buffer for 3 min before stimulation. Each mouse was assayed with 2–4 parallel replicates. The superfusate was collected on 96-well plates in 10 s (~130 µl) fractions, OptiPhase Supermix scintillation cocktail (100 µl/well) was added, and radioactivity was measured with liquid scintillation counting (1 min per well; 1450 MicroBeta TriLux).

[^{3}H]dopamine release data were analyzed using R 2.15.2 (R Core Team). Data were plotted as counts per minute (CPM) versus fraction number, and basal release was calculated for each fraction by single exponential decay from fractions collected before and after the stimulated release peak. Fractions exceeding basal release by 15 % or more were summed to give the amount of nicotine-stimulated release above baseline. Basal release during the first fraction was used as an overall measure of basal release.

4.6.6. BDNF ELISA (Study III)

To study the effects of long-term treatments with levodopa and nicotine on the BDNF, BDNF protein levels in the striatum and the prefrontal cortex (PFC) were measured at the end of the drug treatments (see Figure 10). Mice were killed by cervical dislocation 4 h after the last levodopa administration, and samples of the striatum and PFC were dissected on ice and stored at -80 °C until use. Extraction of proteins was done essentially as described by Rantamäki et al. (2013). A commercial ELISA kit (BDNF Emax® ImmunoAssay; Promega, Madison, WI, USA) was used. The manufacturer's instructions were followed except for two modifications. First, Optacoat™ (ALerCHEK, Springvale, ME, USA) was used as the coating buffer, and second, the samples were transiently acidified and neutralized (Rantamäki et al., 2013) without first diluting them in DPBS, followed by dilution with Block & Sample 1X Buffer. Data were expressed as percentage of the mean of the unlesioned hemisphere of the control group.

4.7. Statistical methods

All data are represented as mean ± SEM or as box plots with median, quartiles, range, and distribution shown. Outliers were removed from all data using the Tukey Box-Plot method (Tukey, 1977). Group differences were analyzed with unpaired two-tailed Student's t-tests, analyses of variance (ANOVA), or repeated measures ANOVA (RM-ANOVA) as appropriate. The Bonferroni *post hoc* correction was used for multiple comparisons after ANOVA, and multiplicity-adjusted P values are reported. For RM-ANOVA, missing values were interpolated with linear regression, and the assumption of sphericity was tested with Mauchly's test and corrected with the Greenhouse-Geisser correction. Pearson's correlation coefficients were calculated for correlation analyses. Differences in postoperative mortality and successful lesioning were analyzed with Pearson's Chi-Square tests. Parallel non-parametric statistical analyses of integrated dyskinesia scores were performed with Mann-Whitney U tests, Kruskal-Wallis ANOVA with Dunn's post hoc correction, or by calculation of Spearman's correlation coefficients (see 6.1.2. below for the rationale). The level of statistical significance was set at $P < 0.05$. Statistical analyses were performed with GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA), with the exception of three-way RM-ANOVA and all sphericity tests and corrections, which were performed with IBM SPSS Statistics 24 (IBM, Armonk, NY, USA).

5. Results

5.1. Characterization of the 6-OHDA lesion models in wild-type mice

See Figure 11 for representative brain sections of mice lesioned with intrastriatal and intra-MFB 6-OHDA injections. 4–5 weeks after 6-OHDA injections into the dorsal striatum, a partial loss of TH-positive immunostaining in the dorsal striatum (35 ± 1 % remaining) and the SNC (40 ± 10 % remaining) was observed densitometrically, with the ventral striatum being less affected (Study II, n = 5 female mice). Similarly, stereological counting in the SNC showed a partial loss of dopaminergic neurons 3–4 weeks after intrastriatal 6-OHDA injections, with 17 ± 3 % of cell bodies remaining in the SNCD and 19 ± 3 % in the SNCM (Study I, n = 7 mice, 5 female).

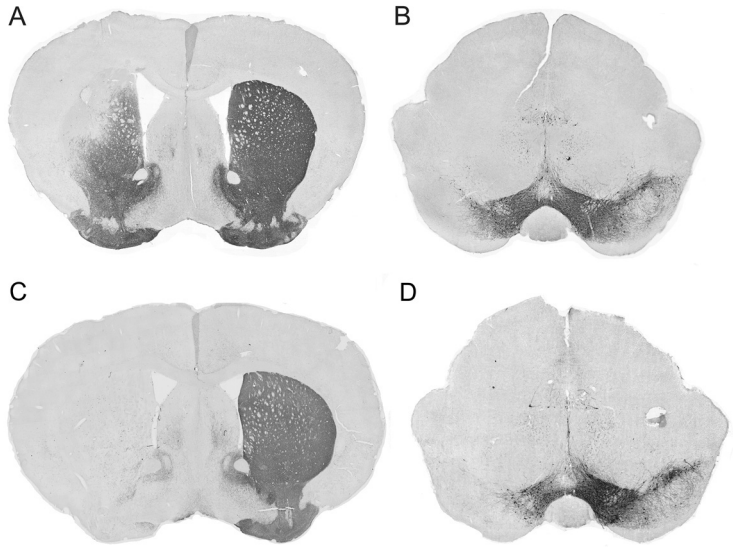

Figure 11: Representative brain sections, immunostained for tyrosine hydroxylase, from mice lesioned with stereotactic 6-OHDA injections. Two injections of 6 μg 6-OHDA into the dorsal striatum resulted in partial nigrostriatal denervation (Study II). Partial losses of immunostaining in the dorsal striatum (**A**) and the dopaminergic areas of the ventral midbrain (**B**) were observed, the latter being mostly restricted to the substantia nigra. Note that in Study I intrastriatal 6-OHDA was administered slightly more medially. One injection of 3 μg 6-OHDA into the medial forebrain bundle resulted in much more severe nigrostriatal as well as mesolimbic denervation (Study I). A practically total loss of immunostaining was observed in both the dorsal and ventral striatum (**C**), along with a marked loss of immunostaining in the ventral midbrain (**D**) that extended also to other dopaminergic areas. Sections were cut at ca. +0.8 mm (A, C) or -3.3 mm (B, D) from the bregma.

In the first experiments with intra-MFB 6-OHDA injections, variable results were obtained (see Study I, Supplement), likely reflecting the challenging procedure. Subsequent studies confirmed that successful intra-MFB 6-OHDA injections resulted in a practically total abolishment of TH-positive immunostaining in both the dorsal and the ventral striatum (not reliably quantifiable with densitometry), along with a marked loss of immunostaining in the SNC (9 ± 2 % remaining 20 weeks after the lesion in Study III; n = 10 female mice). Similarly, HPLC measurements showed a near-total loss of dopamine in the dorsal striatum (1.8 ± 0.4 % remaining 15 weeks lesioning in Study I; n = 5 female mice), and stereological counting found a near-total loss of dopaminergic cell bodies in the SNCD (6 ± 1 % remaining, Study I; n = 8). Surprisingly, however, neurodegeneration in the SNCM was only partial in these mice (45 ± 5 % remaining, n = 8).

5.2. Effects of α5 gene deletion in mouse models of Parkinson's disease

5.2.1. Effects of α5 gene deletion on dopaminergic denervation and drug-induced rotation (Study I)

To study the consequences of the lack of α5* nAChRs in mouse models of PD, the extent of the nigrostriatal denervation in 6-OHDA-lesioned α5-KO and WT mice was determined by stereological counting of TH-positive cell bodies within the SNCD and the SNCM. The SNC subdivisions were analyzed separately due to their readily apparent difference in lesion extent in MFB-lesioned mice. See Study I, Fig. 1 for images of representative brain sections and examples of delineations of the SNCD and the SNCM. Measurements of drug-induced locomotion were used for behavioral characterization of the dopaminergic lesions as well as for assessment of the effects of amphetamine in intact animals. Results pertaining to LID in α5-KO mice are presented later (5.3.3., Figure 19).

Figure 12 shows the results of stereological cell counting in the intrastriatal 6-OHDA lesion model. Both subdivisions of the SNC had suffered lesions of roughly similar extent. Numbers of remaining cells were estimated with CE 0.14–0.39 for SNCD and CE 0.12–0.28 for SNCM. In the SNCD, no difference was found between WT and α5-KO animals in TH-positive cells remaining (WT 16.6 ± 2.9 % vs. α5-KO 18.8 ± 2.9 %; two-way ANOVA, main effect of genotype, $F(1,13) = 0.96$, $P = 0.346$; n = 7 WT, 5 female; 10 KO, 7 female). SNCD cell loss tended to be more pronounced in male animals (main effect of sex, $F(1,13) = 4.06$, $P = 0.065$). In the SNCM, in contrast, more TH-positive cells remained in α5-KO animals (WT 18.5 ± 2.9 % vs. α5-KO 26.9 ± 1.5 %; main effect of genotype, $F(1,13) = 6.72$, $P = 0.022$), with similar results for both sexes.

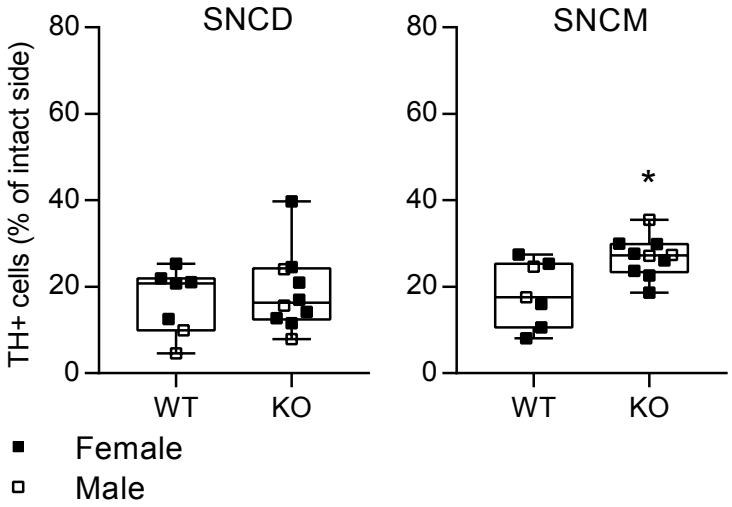

Figure 12. Attenuated dopaminergic denervation in α5-knockout mice. α5-knockout (KO) and wild-type (WT) mice were lesioned with 6-hydroxydopamine injections into the dorsal striatum. Lesion extent was determined 30 days after surgery by stereological counting of tyrosine hydroxylase (TH)-positive cell bodies in the dorsal tier (SNCD) and the medial cluster (SNCM) of the substantia nigra pars compacta. In the SNCM, more TH-positive cells remained in α5-KO mice. * $P < 0.05$, two-way ANOVA. $n = 7$ WT, 10 KO.

See Study I, Fig. 2 for the results of drug-induced rotation and locomotion tests in intrastriatally lesioned and intact mice. Among intrastriatally lesioned mice, female α5-KO mice performed fewer ipsilateral rotations after amphetamine administration than female WT mice (two-way RM-ANOVA, main effect of genotype, $F(1,11) = 5.23$, $P = 0.042$; $n = 5$ WT, 8 KO), but there was no statistically significant genotype difference in contralateral rotations after apomorphine administration. In contrast, among male animals there was no genotype difference in amphetamine-induced rotations but α5-KO mice performed more apomorphine-induced rotations than WT mice (two-way RM-ANOVA, main effect of genotype, $F(1,5) = 16.1$, $P = 0.010$; $n = 3$ WT, 4 KO). In intact mice, no genotype difference was observed in the distance travelled after amphetamine administration (two-way RM-ANOVA, main effect of genotype, $F(1,25) = 0.01$, $P = 0.930$; n = 14 WT, 6 female; 13 KO, 10 female), with similar results for both sexes (males: $F(1,9) = 0.22$, $P = 0.652$; females: $F(1,14) = 0.39$, $P = 0.544$; data not shown).

See Study I for the immunohistochemical and rotametry results obtained using the intra-MFB 6-OHDA lesion model. In the pilot experiment (Study I, Supplement; male mice), although lesion success was variable and postoperative mortality was high (52 % WT, 63 % KO), fewer successful severe striatal lesions were observed in surviving α5-KO animals (more spared TH immunoreactivity in α5-KO animals, t-test, $t(18) = 2.12$, $P = 0.048$; $n = 11$

WT, 9 KO). Moreover, α5-KO mice tended to perform fewer rotations after amphetamine administration (two-way RM-ANOVA, main effect of genotype, $F(1,13) = 4.40$, $P = 0.056$; $n = 8$ WT, 7 KO). In the main experiment (Study I, Fig. 3; female mice), a rough estimate (CE 0.25–0.71) suggested no genotype difference in the proportion of TH-positive cells remaining in the lesioned SNCD (WT 6.0 ± 0.9 % vs. α5-KO 8.5 ± 2.2 %; two-way ANOVA, main effect of genotype, $F(1,13) = 1.01$, $P = 0.332$; $n = 8$ WT, 10 KO). In the SNCM, more accurate estimates (CE 0.13–0.24) could be obtained due to a markedly less severe cell loss, and no significant genotype difference was found in cells remaining (WT 45.1 ± 5.2 % vs. α5-KO 36.4 ± 3.7 %; main effect of genotype, $F(1,13) = 2.59$, $P = 0.131$). The number of cells remaining did not differ between nicotine-treated and control mice in either area. Results of HPLC analyses of striatal dopamine levels are not shown, as a significant number of samples were accidentally destroyed, preventing meaningful group comparisons. Female α5-KO mice of the intra-MFB model tended to perform fewer amphetamine-induced rotations than WT mice (two-way RM-ANOVA, main effect of genotype, $F(1,18) = 3.70$, $P = 0.070$; $n = 10$ per genotype).

5.2.2. *Effects of α5 gene deletion on striatal markers of dopaminergic function (Study I)*

Various dopaminergic markers were measured to investigate the effects of the lack of α5* nAChRs on the dopaminergic system. Uptake of dopamine into striatal synaptosomes (Figure 13) was decreased in intact α5-KO mice, suggesting reduced DAT function (main effect of genotype, $F(1,22) = 6.62$, $P = 0.017$; $n = 13$ WT, 5 female; 13 KO, 4 female). Dopamine uptake was larger in male than female animals (main effect of sex, $F(1,22) = 6.30$, $P = 0.020$), but the genotype difference was similar in both sexes.

No genotype differences were found in other dopaminergic markers (see Study I, Fig. 4). When striatal DAT and pDAT protein levels were measured with Western blotting, no statistically significant genotype or sex differences were found ($n = 6$ WT, 4 female; 8 KO, 5 female). Similarly, no statistically significant genotype or sex differences were found in striatal tissue concentrations of dopamine or its metabolites, measured with HPLC from intact mice ($n = 9$ WT, 5 female; 13 KO, 7 female). Finally, the striatal expression of dopamine D1 and D2 receptor mRNA was measured with qPCR from intrastriatally lesioned mice. The dopaminergic lesion significantly reduced both D1R and D2R mRNA expression (three-way ANOVA, main effect of hemisphere, D1R: $F(1,32) = 25.9$, $P < 0.001$; D2R: $F(1,32) = 21.2$, $P < 0.001$; $n = 8$ WT, 5 female; 12 KO, 8 female). However, expression did not differ between genotypes. D1R mRNA expression was larger in male than female animals (main effect of sex, $F(1,32) = 21.2$, $P < 0.001$). The lesion-induced decrease in D1R and possibly D2R mRNA was greater in male animals (sex × hemisphere interactions, D1R: $F(1,32) = 14.6$, $P < 0.001$; D2R: $F(1,32) = 3.77$, $P = 0.061$).

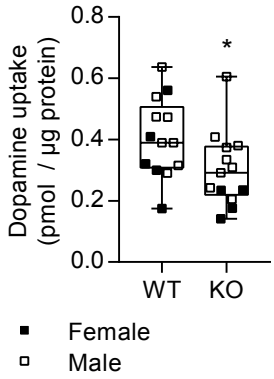

Figure 13. Reduced synaptosomal dopamine uptake in α5-knockout (KO) mice when compared to wild-type (WT) mice. * $P < 0.05$, two-way ANOVA. Uptake was larger in male mice ($P < 0.05$). $n = 13$ WT, 13 KO, assayed in triplicate.

5.3. Levodopa-induced dyskinesia in mouse models of Parkinson's disease

5.3.1. Characterization of levodopa-induced dyskinesia in mice (Studies I–III)

The majority of the mice lesioned with 6-OHDA and treated with repeated systemic administration of levodopa and benserazide (41 out of 49 mice in total, 84 %) developed abnormal involuntary movements, considered to model LID. See Figure 14 for examples of such abnormal movements. The abnormal movements typically developed within days, and breaks in levodopa administration during weekends or washouts had no discernible effect on their expression. As previously reported (Lundblad et al., 2004; Francardo et al., 2011), some animals did not develop LID, and mice of the intra-MFB model were much more sensitive to levodopa than mice of the intrastriatal model. The latter was reflected by lower levodopa doses needed to induce dyskinesia, the dyskinesia being considerably more severe, and a smaller proportion of mice remaining non-dyskinetic (3 out of 33 mice, 9 %, vs. 5 out of 16 mice, 31 %).

Figure 15 shows baseline dyskinesia scores, obtained using the novel criteria in Table 6, in wild-type mice after 2–3 weeks of levodopa administration. Note that the data were combined from different experiments (see Figure 10) using similar methodology (but see below). Figure 15A shows axial, orolingual and forelimb dyskinesia scores as well as total dyskinesia scores (a measure of global dyskinesia of an animal) elicited by different doses of levodopa in female mice lesioned with intra-MFB 6-OHDA injections (3 mg/kg, Study I; 4.5 mg/kg, amantadine study; 6 mg/kg, Study III; $n = 8–17$ per group). Figure 15B shows, for comparison, dyskinesia scores elicited by 30 mg/kg levodopa in mice lesioned with intrastriatal 6-OHDA injections (Study II control group; $n = 5$, 3 female). Mice treated

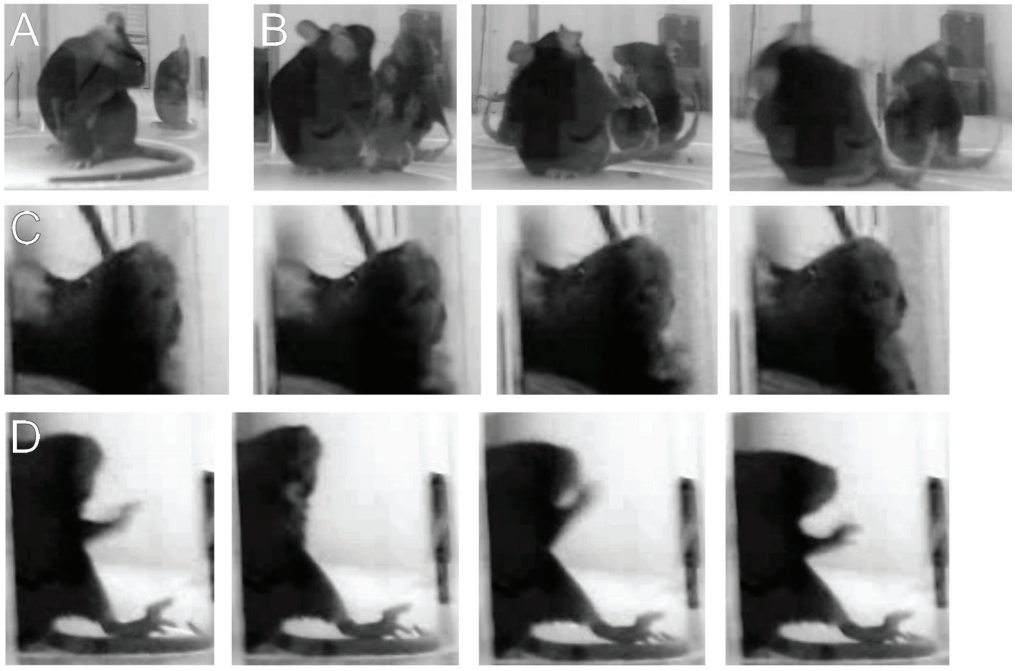

Figure 14. Representative images of levodopa-induced abnormal involuntary movements. Images were captured from video recordings of mice lesioned with intra-MFB 6-OHDA injections. **A**: Moderate axial dyskinesia (2 points), deviation of the head/trunk in a sitting position. **B**: Severe axial dyskinesia (4 points), severe twisting of the head/trunk followed by a loss of balance. **C**: Orolingual dyskinesia, purposeless chewing motions and tongue protrusion. Orolingual dyskinesia can also manifest as repeated biting of the contralateral side/limbs. **D**: Severe forelimb dyskinesia (4 points), repetitive large swings of the contralateral forelimb. For representative video material of severe dyskinesia (4 points) see http://urn.fi/URN:ISBN:978-951-51-4855-1

with levodopa expressed all dyskinesia subtypes in a time-dependent manner (2-way RM-ANOVA, main effect of time, axial: $F(3.0,103.3) = 20.0$, $P < 0.001$; orolingual: $F(4,136) = 15.1$, $P < 0.001$; forelimb: $F(3.1,105.6) = 8.34$, $P < 0.001$; total: $F(3.1,105.5) = 26.2$, $P < 0.001$). Maximal dyskinesia appeared within 20 minutes and continued for approximately one hour before gradually disappearing.

Dyskinesia scores also exhibited some dose-dependency (Figure 15A). Orolingual, forelimb, and total dyskinesia scores were statistically significantly affected by the levodopa dose (2-way RM-ANOVA, main effect of dose, orolingual: $F(2,34) = 6.38$, $P = 0.005$; forelimb: $F(2,34) = 24.2$, $P < 0.001$; total: $F(2,34) = 11.0$, $P < 0.001$). Post hoc tests show that the dose of 6 mg/kg elicited higher orolingual, forelimb and total dyskinesia scores than the

doses of 4.5 mg/kg (orolingual, P = 0.005; forelimb, P < 0.001; total, P < 0.001) or 3 mg/kg (orolingual, P = 0.039; forelimb, P = 0.001; total, tendency P = 0.065). In contrast, similar or even lower orolingual and forelimb dyskinesia scores were elicited by the dose of 4.5 mg/

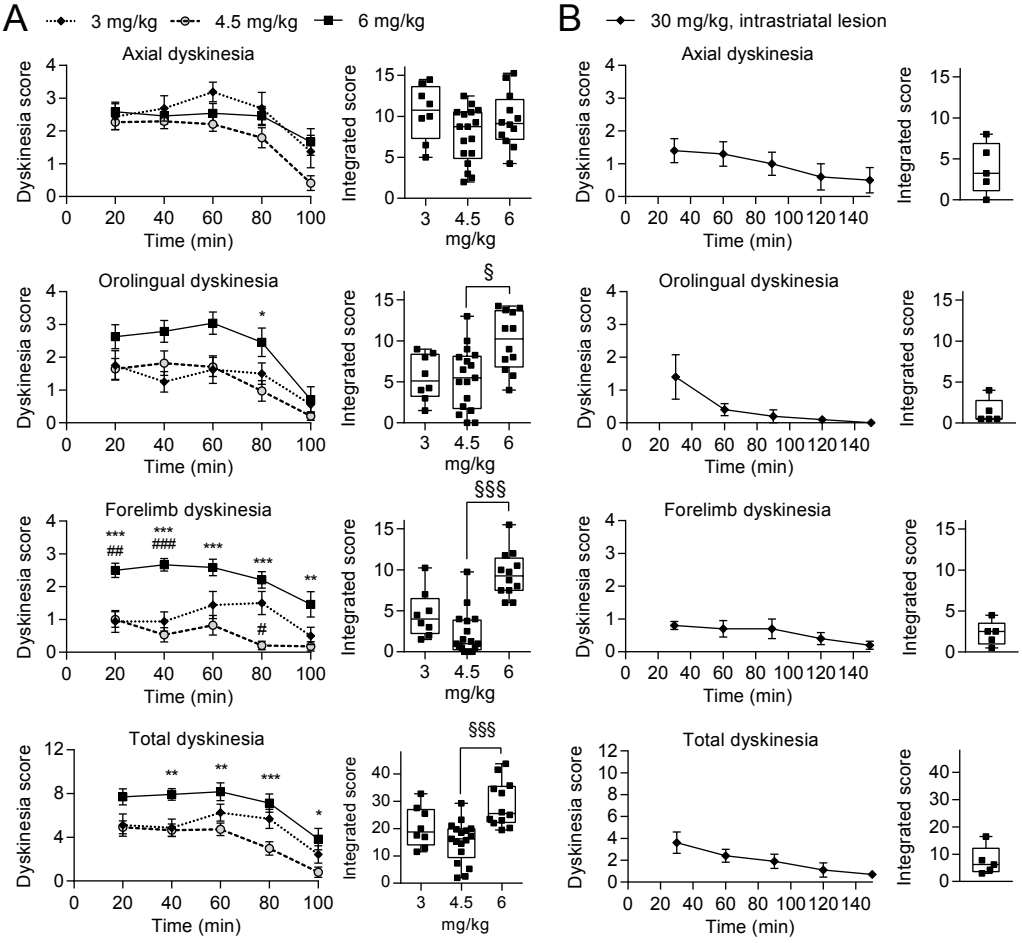

Figure 15. Levodopa-induced dyskinesia in wild-type mice. Mice were lesioned with intra-MFB (**A**) or intrastriatal (**B**) 6-OHDA and administered levodopa and benserazide (s.c.) for 2–3 weeks. The severity of axial, orolingual, and forelimb dyskinesia after levodopa administration was then assessed. Total dyskinesia refers to the sum of the subtype scores. Line graphs show mean scores ± SEM. Box plots show integrated dyskinesia scores (area under the curve). **A**: Considerable dyskinesia was observed in MFB-lesioned mice. Orolingual and forelimb dyskinesia induced by 6 mg/kg levodopa was assessed as more severe than that induced by lower doses, but the doses of 3 and 4.5 mg/kg did not differ, possibly due to methodological discrepancies (see text for details). All doses induced similar axial dyskinesia. *n* = 12 (6 mg/kg), 17 (4.5 mg/kg), 8 (3 mg/kg). **B**: Markedly less severe dyskinesia was observed in striatally lesioned mice (*n* = 5). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to 4.5 mg/kg, # $P < 0.05$, ### $P < 0.001$ compared to 3 mg/kg, two-way RM-ANOVA with Bonferroni's *post hoc* tests; § $P < 0.05$, §§§ $P < 0.001$ compared to 4.5 mg/kg, Kruskall-Wallis ANOVA with Dunn's *post hoc* tests.

kg compared to 3 mg/kg. Note, however, some methodological discrepancies at the dose of 3 mg/kg (see 6.1.2.). High axial dyskinesia scores were elicited by all levodopa doses, with no differences between doses (2-way RM-ANOVA, main effect of dose, $F(2,34) = 2.59$, $P = 0.090$). Non-parametric statistical analysis confirmed that the dose of levodopa had a statistically significant effect on orolingual, forelimb, and total dyskinesia scores but not on axial dyskinesia scores (Kruskal-Wallis ANOVA, axial: $H = 3.45$, $P = 0.178$; orolingual: $H = 8.74$, $P = 0.013$; forelimb: $H = 21.7$, $P < 0.001$; total: $H = 15.3$, $P < 0.001$).

The final recordings of Studies II and III were scored with both the novel criteria and the criteria of Lundblad et al. (2004), using a truncated version in Study III as interruptibility was not assessed. In both cases, scores obtained with the different methods were highly correlated (Study II: Pearson's $r = 0.96$, $P < 0.001$; Spearman's $r = 0.92$, $P < 0.01$; $n = 11$; Study III: Pearson's $r = 0.89$, $P < 0.001$; Spearman's $r = 0.78$, $P < 0.01$; $n = 12$).

5.3.2. Effects of drug treatments on levodopa-induced dyskinesia (Studies II–III)

Figure 16 shows the development of dyskinesia scores in mice lesioned with intrastriatal 6-OHDA (Study II) and treated chronically with levodopa and either nicotine-containing or normal drinking water (initiated simultaneously). Average nicotine intake at 300 µg/ml was 36 ± 10 mg/kg/day. The nicotine treatment inhibited the development of all LID subtypes, particularly after the treatment had been continuing for several weeks. At the time of the final measurements, mean dyskinesia scores were considerably lower in nicotine-treated animals (ca. −50% for axial dyskinesia, −80% for orolingual dyskinesia, −70% for forelimb dyskinesia).

In statistical analysis, total dyskinesia only had a tendency to be attenuated in nicotine-treated animals (2-way RM-ANOVA, main effect of treatment, $F(1,9) = 4.55$, $P = 0.062$, $n = 5$ control mice, 3 female; 6 nicotine-treated mice, 4 female). However, subtype-specific analyses revealed statistically significant effects. Forelimb dyskinesia was significantly attenuated (main effect of treatment, $F(1,9) = 7.99$, $P = 0.020$). While the main effect of treatment was not significant for the other subtypes (axial: $F(1,9) = 2.56$, $P = 0.144$; orolingual: $F(1,9) = 3.00$, $P = 0.117$), a significant treatment × time interaction shows that there was a difference between the treatment groups in how axial dyskinesia scores changed over time ($F(4,36) = 3.15$, $P = 0.026$). Although no post hoc comparisons reached significance, the data quite clearly suggest inhibition of axial LID by the nicotine treatment. A tendency along similar lines was observed for orolingual dyskinesia scores (treatment × time interaction, $F(2.2,20.0) = 2.91$, $P = 0.073$). While no group differences reached significance in non-parametric statistical analyses, tendencies suggest attenuation of orolingual (Mann-Whitney U test, $U = 5.0$, $P = 0.076$) and forelimb ($U = 5.5$, $P = 0.089$) dyskinesia as well as total dyskinesia ($U = 5.0$, $P = 0.082$) in nicotine-treated animals but no group difference in axial dyskinesia ($U = 9.0$, $P = 0.329$).

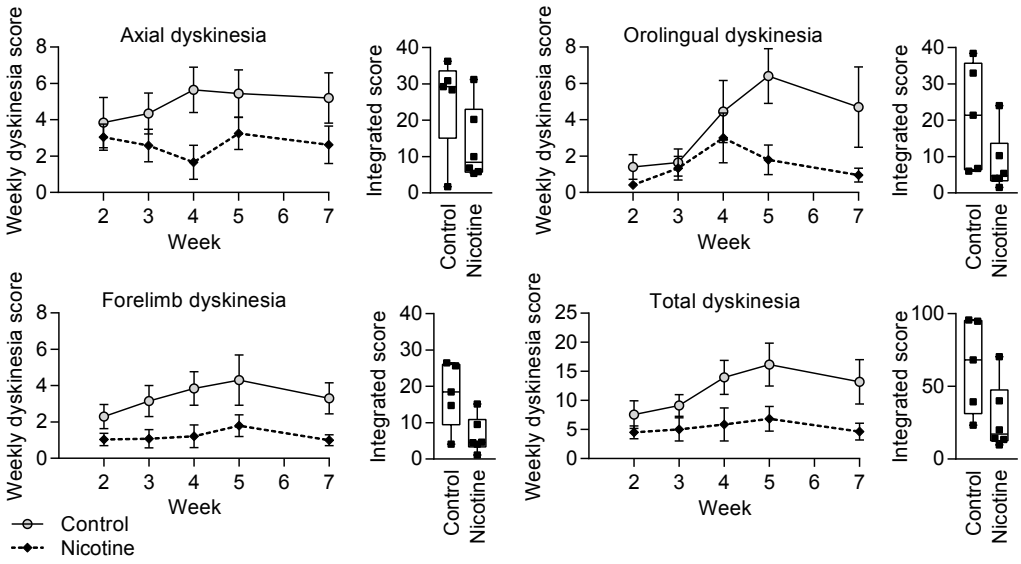

Figure 16. Nicotine treatment inhibited the development of levodopa-induced dyskinesia in mice lesioned with intrastriatal 6-OHDA injections. The mice were chronically administered levodopa (30 mg/kg) and benserazide (12 mg/kg) (s.c.) and nicotine (up to 300 µg/ml) in drinking water. Both treatments were initiated simultaneously. Dyskinesia severity was assessed weekly. Line graphs show mean weekly dyskinesia scores ± SEM. Two-way RM-ANOVA: Axial dyskinesia, treatment × time interaction $P < 0.05$; Orolingual dyskinesia, interaction $P = 0.07$; Forelimb dyskinesia, main effect of nicotine $P < 0.05$; Total dyskinesia, main effect of nicotine $P = 0.06$. Box plots show integrated dyskinesia scores for the entire experiment. Mann-Whitney U test: Orolingual dyskinesia, $P = 0.08$; Forelimb dyskinesia, $P = 0.09$; Total dyskinesia, $P = 0.08$. $n = 5–6$ per group.

Figure 17 shows dyskinesia scores in female mice lesioned with intra-MFB 6-OHDA (Study III) and treated chronically with levodopa and nAChR agonists. The mice were rendered dyskinetic with 2 weeks of levodopa treatment and then administered (s.c.) either escalating doses of AZD0328 or saline. AZD0328 treatment had no effect on total dyskinesia scores (Figure 17A and Study III, Figure 3), with no statistically significant findings ($n = 6$ per group). Subtype-specific analyses revealed only one significant finding, where axial dyskinesia was reduced by the lowest dose of AZD0328 (0.03 mg/kg) 100 min after levodopa injection (2-way RM-ANOVA, treatment × time interaction, $F(4,40) = 3.37$, $P = 0.018$; data not shown). However, the control group appeared to exhibit aberrantly long-lasting axial dyskinesia at that particular date.

After a washout, the mice of Study III were randomly re-assigned to two groups and chronic treatment with either nicotine or vehicle (saccharin-sweetened drinking water) was initiated. Average nicotine intake at 300 µg/ml was 35 ± 10 mg/kg/day. Total dyskinesia scores (Figure 17B) and subtype-specific dyskinesia scores (Study III, Figure 3) were not

lower in nicotine-treated animals. However, while no treatment main effects reached statistical significance, there was a difference between the treatment groups in how total, axial, and possibly forelimb dyskinesia scores changed over time (2-way RM-ANOVA, treatment × time interactions, total: $F(8,80) = 2.97$, $P = 0.006$; axial: $F(8,80) = 3.04$, $P = 0.005$; forelimb: $F(8,80) = 1.93$, $P = 0.067$; $n = 6$ per group). While no post hoc tests reached statistical significance, a possible interpretation is that LID severity was first transiently increased in nicotine-treated animals and then returned to control levels. Non-parametric statistical analysis found no statistically significant group differences. Finally, instances of severe axial dyskinesia (losses of balance) per minute were separately counted, and no differences between treatment groups were observed (data not shown).

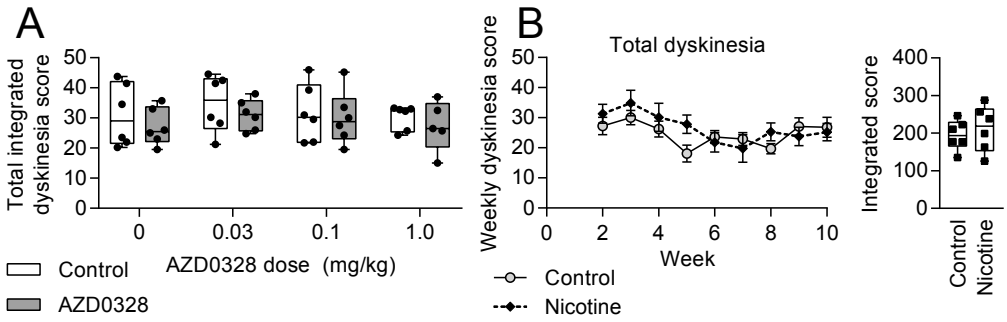

Figure 17. No attenuation of levodopa-induced dyskinesia by nAChR agonists in mice lesioned with intra-MFB 6-OHDA injections. Dyskinesia was pre-established by two weeks of levodopa treatment (6 mg/kg, s.c.). **A**: The α7 receptor partial agonist AZD0328 was administered (s.c.) 30 min before levodopa for 5 days per dose, and dyskinesia severity was assessed on the fifth day. No differences in total dyskinesia between treatment groups were found. **B**: After a washout, chronic levodopa treatment was continued and nicotine treatment in drinking water (up to 300 μg/ml) initiated. Dyskinesia severity was assessed weekly. Nicotine treatment may have transiently increased dyskinesia severity. Two-way RM-ANOVA, treatment × time interaction $P < 0.01$. Shown are integrated total dyskinesia scores for the recording session (A) or mean total dyskinesia scores ± SEM along with integrated scores for the entire experiment (B). $n = 6$ per group.

Figure 18 shows dyskinesia scores in female mice lesioned with intra-MFB 6-OHDA and treated chronically with levodopa and acutely with amantadine (additional study). Doses of 5, 15, and 60 mg/kg amantadine, administered (i.p.) 60 min before levodopa, failed to attenuate LID, with no statistically significant treatment main effects at any dose. Moreover, amantadine pretreatment prolonged dyskinetic behaviors, an effect readily apparent during the recording session. Statistically significant effects on LID were observed at the dose of 60 mg/kg (Figure 18A; 2-way RM-ANOVA, treatment × time interactions, axial: $F(2.7,43.3) = 6.56$, $P = 0.001$; forelimb: $F(5,80) = 3.31$, $P = 0.009$; orolingual: $F(2.5,40.4) = 2.66$, $P = 0.070$; total: $F(2.8,44.7) = 4.52$, $P = 0.009$; $n = 9$ per group). Again, however, no post hoc

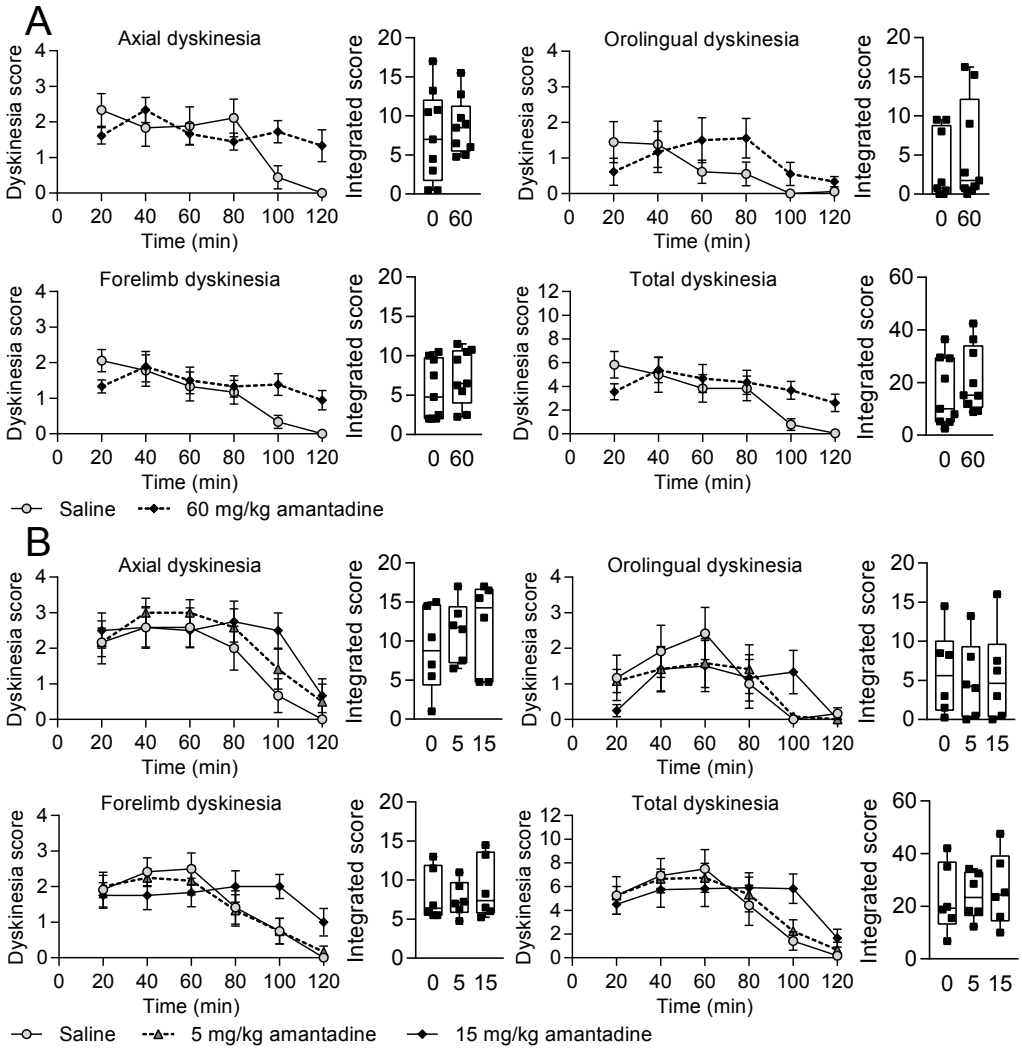

Figure 18. Unattenuated and prolonged levodopa-induced dyskinesia after acute amantadine treatment in mice lesioned with intra-MFB 6-OHDA injections. **A**: The mice were administered levodopa (4.5 mg/kg) for two weeks. At the last day, amantadine (60 mg/kg, i.p.) was administered 60 min before levodopa, and dyskinesia severity was assessed. Amantadine pretreatment failed to reduce dyskinesia and, instead, markedly prolonged dyskinetic behaviors. Two-way RM-ANOVA, treatment × time interactions: Axial dyskinesia, $P = 0.001$; Orolingual dyskinesia, $P = 0.07$; Forelimb dyskinesia, $P < 0.01$; Total dyskinesia, $P < 0.01$. **B**: After a washout and further three weeks of levodopa administration, amantadine (5 or 15 mg/kg, i.p.) was administered 60 min before levodopa, and dyskinesia severity was assessed. Lower dose amantadine pretreatment also failed to reduce dyskinesia scores. The dose of 15 mg/kg had modest prolonging effects. Two-way RM-ANOVA, treatment × time interaction: Forelimb dyskinesia, $P < 0.05$. Line graphs show mean dyskinesia scores ± SEM. Box plots show integrated weekly dyskinesia scores. $n = 9$ (A) or 6 (B) per group.

comparisons reached significance. When lower doses of amantadine were tested, the dose of 15 mg/kg had shorter-lasting but still clearly discernible prolonging effects (Figure 18B), although only the effect on forelimb LID was significant (treatment × time interaction, $F(5,75) = 2.20$, $P = 0.026$; $n = 6$ per group). Non-parametric statistical analysis found no significant group differences in integrated dyskinesia scores. Administration of 60 mg/kg amantadine 0 and 100 min before levodopa was also tested, with very similar results of prolonged and unattenuated LID (data not shown).

5.3.3. Levodopa-induced dyskinesia in α5-knockout mice (Study I)

Figure 19 shows dyskinesia scores in female α5-KO and WT mice lesioned with intra-MFB 6-OHDA (Study I). The mice were rendered dyskinetic with 3 weeks of levodopa treatment, after which chronic treatment with either nicotine or vehicle (saccharin-sweetened drinking water) was initiated. The average intake of nicotine at 300 μg/ml was 31 ± 6 mg/kg/day. Total dyskinesia scores were lower in α5-KO mice than in WT mice (three-way RM-ANOVA, main effect of genotype, $F(1,14) = 12.3$, $P = 0.004$; $n = 4–5$ per genotype and treatment). Non-parametric statistical analysis confirmed the lower dyskinesia scores in α5-KO mice (Mann-Whitney U test, $U = 9.0$, $P = 0.004$). In addition, there was a difference between nicotine-treated α5-KO and WT mice in how dyskinesia scores changed over time (genotype × treatment × time interaction, $F(3.5,48.5) = 3.14$, $P = 0.028$; genotype × time interaction only in nicotine-treated animals, $F(3.2,22.3) = 4.73$, $P = 0.010$). Although no post hoc comparisons reached statistical significance, a possible interpretation is that LID severity decreased over time in nicotine-treated WT but not α5-KO mice. The effect of nicotine treatment was not significant in non-parametric statistical analysis.

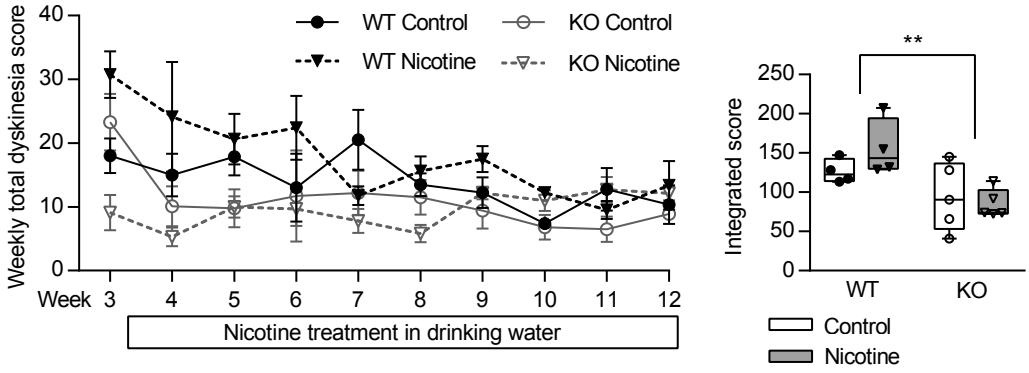

Figure 19. Levodopa-induced dyskinesia in α5-knockout (KO) and wild-type (WT) mice. Mice lacking α5* nicotinic receptors developed less severe LID than wild-type mice (three-way RM-ANOVA, $P < 0.01$, $n = 8$ WT, 10 KO, female). In nicotine-treated mice, dyskinesia severity may have been reduced over time in WT but not α5-KO mice (genotype × treatment × time interaction, $P < 0.05$; genotype × time interaction in nicotine-treated animals, $P = 0.01$; $n = 4–5$ per group). Line graph shows mean weekly dyskinesia scores ± SEM. Box plots show integrated dyskinesia scores for the entire experiment. ** $P < 0.01$, difference between WT and KO, Mann-Whitney U test.

5.3.4. *Effects of antidyskinetic nicotine treatment on striatal dopamine release (Study II)*

To determine the effects of dopaminergic denervation and chronic nicotine treatment on dopaminergic nerve terminals and their presynaptic nAChRs, basal and nicotine-stimulated [^{3}H]dopamine release was measured from striatal synaptosomes. Synaptosomes were separately prepared from the dorsal and ventral striatum of both hemispheres of mice lesioned with intrastriatal 6-OHDA. Nicotine-stimulated release was separated to α4(non-α6)β2*-mediated and α6β2*-mediated release with CtxMII and normalized to basal release.

See Study II, Fig. 3 for the full results. Basal [^{3}H]dopamine release from samples of the lesioned dorsal striatum was reduced 4–5 weeks after lesioning to 37 ± 5% of the intact side (t-test, $t(10) = 8.15$, $P < 0.001$; $n = 6$, all female). In animals assayed after 7–8 weeks of chronic treatment with levodopa and either nicotine or water (15–16 weeks after lesioning), basal release from samples of the lesioned dorsal striatum remained significantly reduced (74 ± 10% of the intact side; two-way ANOVA, main effect of hemisphere, $F(1,17) = 5.24$, $P = 0.035$; $n = 5$ control mice, 3 female; 6 nicotine-treated mice, 4 female). In samples of the lesioned ventral striatum, 4–5 weeks after lesioning basal [^{3}H]dopamine release was reduced to 61 ± 9% of the intact side ($t(10) = 3.25$, $P = 0.009$). In animals assayed after the chronic drug treatments, basal release from the lesioned ventral striatum was no longer significantly reduced (main effect of hemisphere, $F(1,18) = 2.75$, $P = 0.115$). In particular, in nicotine-treated animals basal release from the lesioned ventral striatum was comparable to the intact hemisphere (main effect of treatment, $F(1,18) = 7.93$, $P =$

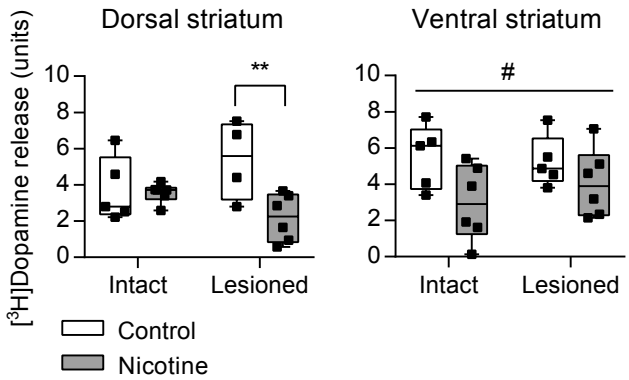

Figure 20. Chronic nicotine treatment reduced synaptosomal [^{3}H]dopamine release mediated by α6β2* nicotinic receptors. Mice were lesioned with intrastriatal 6-OHDA injections and administered levodopa and either nicotine-containing or normal drinking water for 7–8 weeks. α6β2*-mediated release was calculated as the difference between total and α-conotoxin MII-resistant release. ** $P < 0.01$, difference to control, # $P < 0.05$, main effect of treatment, two-way ANOVA with Bonferroni's *post hoc* comparisons. $n = 4$–6 mice, assayed in duplicate.

0.011; treatment × hemisphere interaction, F(1,18) = 3.60, P = 0.074). nAChR-mediated normalized [^{3}H]dopamine release was not affected by the dopaminergic lesion. The chronic nicotine treatment had no statistically significant effect on α4(non-α6)β2*-mediated release in either brain area. However, as shown in Figure 20, nicotine treatment significantly decreased α6β2*-mediated [^{3}H]dopamine release in the lesioned dorsal striatum (main effect of treatment, F(1,17) = 6.61, P = 0.020; treatment × hemisphere interaction, F(1,17) = 5.39, P = 0.033) and in both the intact and the lesioned ventral striatum (main effect of treatment, F(1,18) = 7.93, P = 0.028). When non-normalized nAChR-mediated [^{3}H] dopamine release was analyzed, release was diminished in the lesioned hemisphere, but the nicotine treatment had no statistically significant effects (data not shown).

5.3.5. *Effects of levodopa and nicotine treatments on BDNF levels (Study III)*

BDNF protein levels in the striatum and the PFC (Figure 21) were measured from female mice lesioned with intra-MFB 6-OHDA and treated with levodopa and nAChR agonists. No difference in BDNF levels between the lesioned and intact hemispheres was observed in either brain area. Nicotine treatment had no effect on striatal BDNF, but BDNF levels in the PFC were decreased in nicotine-treated animals (two-way ANOVA, main effect of treatment, F(1,20) = 9.06, P = 0.007; n = 6 per group). For analysis of correlation between striatal BDNF and dyskinesia severity, control and nicotine groups were combined as neither dyskinesia severity nor striatal BDNF levels differed between groups. BDNF levels

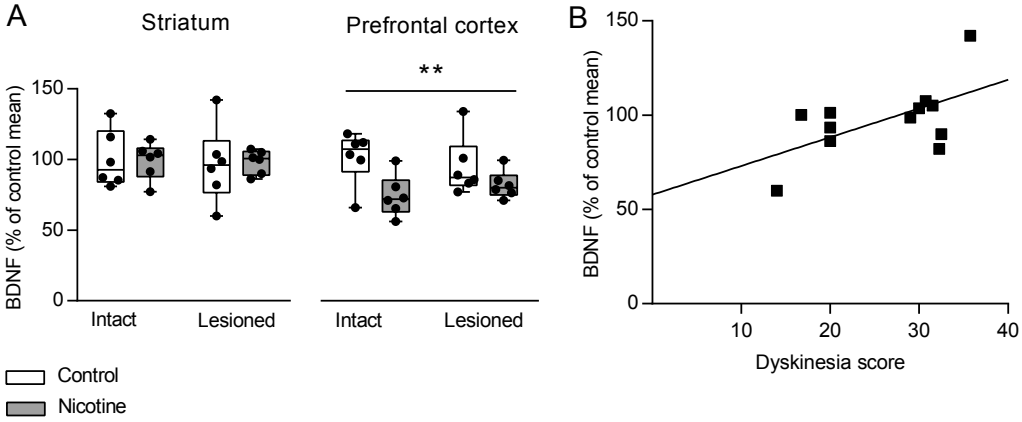

Figure 21. Brain-derived neurotrophic factor (BDNF) protein levels in chronically drug-treated mice previously lesioned with intra-MFB 6-OHDA. **A**: No interhemispheric differences in BDNF protein levels of the striatum and the prefrontal cortex after 15 weeks of levodopa administration. After 10 weeks of chronic nicotine administration BDNF levels were reduced in the prefrontal cortex. **B**: BDNF levels of the lesioned striatum were positively correlated with dyskinesia severity (Pearson's r = 0.582, P < 0.05). n = 6 per group. ** P < 0.01, main effect of treatment, two-way ANOVA.

in the lesioned striatum had a positive linear correlation with weekly total dyskinesia scores at the time of the last dyskinesia measurement (Pearson's r = 0.58, $P = 0.047$, $n = 12$). Non-parametric Spearman's correlation was not statistically significant ($r = 0.40$, $P = 0.198$). No other statistically significant correlations between dyskinesia scores and BDNF levels were found (data not shown).

5.4. Impacts of pre-experimental handling and improved postoperative care (Study IV)

The gradual handling protocol led to what appeared to be obvious and significant reductions in aggressive and escape behaviors. The handled mice exhibited a marked lack of aversion towards the researcher and accepted restraint much more easily than unhandled mice of the same strain. This led to the facilitation of many experimental procedures such as hand feeding during postoperative care, drug administration by injection, euthanasia by cervical dislocation, and handling in general. It is also very likely that this led to a concurrent reduction in animal stress (see Discussion of Study IV). However, the effects of the pre-experimental handling were not studied in a systematic and controlled manner.

Average postoperative mortality was greatly reduced, decreasing from 71 % to 14 % (Pearson Chi-Square test, $\chi^2(1) = 27.8$, $P < 0.001$), after the introduction of the systematic welfare scoring along with the majority of the other refinements described in Study IV. Concurrently, improvements in surgical procedures increased the average rate of successful abundant lesions from 46% to 81% ($\chi^2(1) = 7.45$, $P = 0.006$). Note that these figures include only wild-type animals and that unsuccessfully lesioned animals were not included in the mortality figures. See Study IV for more details on the postoperative mortality and lesion success in the various experiment series.

6. Discussion

6.1. Methodological considerations

6.1.1. Mouse models of Parkinson's disease

The methods for modeling PD in mice can be broadly divided into genetic and neurotoxin models. Genetic models include knockout and transgenic mutations in various PD-associated genes, and although in the future they might replace neurotoxin models particularly in neuroprotection studies, they have been hampered by a lack of dopaminergic neurodegeneration (Dawson et al., 2010). Neurotoxin models are based on toxins such as 6-OHDA, MPTP, rotenone, or paraquat that are relatively selective in destroying dopaminergic neurons (Bové and Perier, 2012). The three latter toxins can be administered systemically to induce bilateral parkinsonism. The metabolite of MPTP, MPP^+, inhibits the mitochondrial complex I and results in e.g., the generation of reactive oxygen species and activation of apoptotic pathways (Bové and Perier, 2012). In mice, acute and chronic MPTP administration can be used to induce severe or partial neurodegeneration, respectively, while in non-human primates chronic administration regimes can be tailored to induce neurodegeneration of required degree. Rotenone is another mitochondrial toxin which also impairs microtubule formation, while paraquat toxicity is mediated by reactive oxygen species formation (Bové and Perier, 2012). The latter two are less commonly used, but may have some advantages such as α-synuclein aggregation that is not seen in the 6-OHDA or acute MPTP mouse models (Bové and Perier, 2012). 6-OHDA crosses the blood-brain barrier poorly and is administered directly into the nigrostriatal pathway, either into the SNC, the MFB, or the striatum (Bové and Perier, 2012). In the present studies, the intra-MFB and intrastriatal models were used.

6-OHDA is thought to enter catecholaminergic neurons through the DAT, there produce reactive oxygen species *via* MAO activity and/or auto-oxidation, and thus induce toxic oxidative stress (Blum et al., 2001). Unilateral administration to induce hemiparkinsonism is almost always used due to very poor survival after bilateral administration. Striatal 6-OHDA administration results in delayed retrograde neurodegeneration within 1–3 weeks, while administration into the MFB results in prompt neurodegeneration within a few days, although in both cases full cell loss can take several weeks (Bové and Perier, 2012). While nigrostriatal neurodegeneration is partial and dose-dependent in the intrastriatal model, neurodegeneration in the intra-MFB model is usually near-total and, furthermore, not restricted to the nigrostriatal pathway. See Figure 11 for examples from the present studies contrasting the relatively restricted nigrostriatal denervation in the intrastriatal model with the near-total loss of both nigrostriatal and mesolimbic innervation in the intra-MFB model. Both lesion models result in quantifiable unilateral motor deficits such as rotation induced by dopaminergic drugs and impaired contralateral limb use (Bové and Perier, 2012). The intrastriatal model has several practical advantages, including the possibility of controlling the lesion by adjusting the 6-OHDA dose and injection site(s) and much better

postoperative survival. However, the intra-MFB model also confers substantial advantages, including more pronounced motor deficits and a higher sensitivity to levodopa-induced dyskinesia (Bové and Perier, 2012).

The challenges of the intra-MFB 6-OHDA model are strikingly demonstrated by the outcomes of the first intra-MFB lesions performed during the present studies (Study I pilot). First, many unsuccessful lesions were observed in surviving animals. Moreover, postoperative mortality was very high, with 52 % of all operated animals and as many as 71 % of successfully lesioned animals lost (only wild-types included), mainly due to euthanasia in response to severe weight loss. These unsatisfactory outcomes reflect both the challenges of targeting a small brain area and, most importantly, the major impact on eating, drinking, and general wellbeing by the near-total dopaminergic denervation, even when applied unilaterally. However, both of these challenges were successfully resolved. In subsequent studies, improved stereotactic protocols, along with accumulating experience of the researcher performing and supervising the surgeries, led to a much higher proportion of successfully lesioned animals. Concurrently, extensively improved postoperative care dramatically reduced postoperative mortality (14 % across all subsequent studies in wild-type mice). The pre-operative gradual handling of the mice performed in some studies also greatly facilitated postoperative care procedures such as feeding by hand (and, in general, all experimental procedures). Besides the refinement of animal wellbeing, these outcomes demonstrate that acceptable postoperative survival after intra-MFB 6-OHDA lesioning is achievable but requires the expending of considerable effort.

A separate and more complex question is the relative suitability of the two 6-OHDA models as models of human PD. On one hand, the symptoms of PD appear and a diagnosis can be made when already roughly 50 % of SNC dopaminergic neurons and 70–80 % of striatal dopamine are lost (Bernheimer et al., 1973; Fearnley and Lees, 1991). In late-stage patients up to 90 % of dopaminergic cells in parts of the SNC and 99 % of dopamine in the putamen can be lost (Bernheimer et al., 1973; Fearnley and Lees, 1991; Hall et al., 2014). Thus, intrastriatal 6-OHDA administration arguably models early or moderate PD, while intra-MFB 6-OHDA administration could be considered to model late-stage PD. On the other hand, even in advanced PD the near-total losses are restricted to certain areas of the SNC and the dorsal striatum, and denervation within the mesolimbic pathway is much less extensive (Bernheimer et al., 1973; Fearnley and Lees, 1991; Hall et al., 2014). Unfortunately, the contributions of mesolimbic or region-specific denervation to parkinsonian pathophysiology are not well-understood.

While both 6-OHDA models have been extensively used in preclinical research, they may have differing applications. For instance, Bové and Perier (2012) suggest that the intra-MFB model is more suitable for studying the consequences of denervation and symptomatic treatments, while the intrastriatal model can be used to study PD pathogenesis and neuroprotective treatments. However, it should be noted that the intra-MFB model may in

fact not be suitable for the study of all potential symptomatic treatments. Using the intra-MFB model to study treatments that could be mediated in significant part by the remaining striatal dopamine terminals could lead to discarding some that would be effective in early PD. An example could be nAChR agonists, which likely exert their antidyskinetic effects in part through dopamine terminal nAChRs and indeed appear to be less effective in conditions of severe dopaminergic denervation, as seen here as well as in a number of previous studies (see 6.3.1. below). Finally, another separate but potentially highly significant question is the overall translational validity of a neurotoxin rodent model of PD characterized by rapid unilateral neurodegeneration, given that human PD is slowly progressing, bilateral, and only attributable to exogenous toxins in a very limited proportion of cases (although a few intriguing findings do suggest the presence of endogenous 6-OHDA in humans; see Bové and Perier, 2012). This issue will not be further discussed here, but see 6.2.2. below for some considerations based on the present findings.

6.1.2. Mouse model of levodopa-induced dyskinesia

The mouse model of LID, building on the unilateral 6-OHDA model of PD, was originally described by the Cenci laboratory (Lundblad et al., 2004; Lundblad et al., 2005; Cenci and Lundblad, 2007; Francardo et al., 2011). Lesioning with either intrastriatal or intra-MFB 6-OHDA can be used, but intra-SNC 6-OHDA was found unsuitable (Francardo et al., 2011). Further developments in MFB injection coordinates and levodopa and benserazide dosing that were followed in the present studies were described by Thiele et al. (2011). As expected, clearly abnormal unilateral movements, considered to model human LID, were rapidly induced in most 6-OHDA-lesioned animals by repeated levodopa administration. Pauses in levodopa administration in primed animals had no effect on LID, in line with the literature (Nadjar et al., 2009).

The mouse model of LID was applied as previously described, with the major exception that the dyskinetic behaviors were quantified with a modified scoring method. The modified scoring method was developed due to observations that the previously published methods (Lundblad et al., 2004; Thiele et al., 2011) appeared not entirely suitable for quantifying severe dyskinesia exhibited by MFB-lesioned mice from video recordings. The original and most widely used scoring method, developed for rats and validated also for mice, assesses dyskinetic behaviors on the basis of frequency (as a proportion of time) and interruptibility (Winkler et al., 2002; Lundblad et al., 2004; Lundblad et al., 2005). However, in the present studies the axial dyskinesia exhibited by the majority of MFB-lesioned mice was continuous and non-interruptible while still showing individual differences in spatial severity ("amplitude"). Various additional criteria for dyskinesia amplitude have been used by different researchers (Cenci and Lundblad, 2007; Huang, Grady, and Quik, 2011), but at the initiation of the present studies only the frequency-based method had been validated for mice. The present scoring method can be seen as measuring both the frequency and the amplitude of orolingual and forelimb dyskinesia, but mainly the amplitude of axial dyskinesia due to the aforementioned propensity for continuous axial dyskinesia.

Interruptibility was not measured due to axial dyskinesia being, if anything, exacerbated by sudden noises or movements. Contralateral rotations, considered "locomotive dyskinesia" in earlier rodent studies, were not included as they are now thought to not reliably reflect dyskinesia (Cenci and Lundblad, 2007).

As mentioned, video recording instead of live assessment was utilized in the present studies. The vast majority of studies utilizing the original scoring method, including those where they were established and validated (Lundblad et al., 2004; Lundblad et al., 2005), have relied on live rating. This might in part explain the challenges in adapting the previously published scoring criteria to the present experiments. Video recording has the advantages of better documented data and the possibility for more detailed (e.g., slow-motion) analysis and reanalysis. On the other hand, video recording has the disadvantage of a static viewing angle, which was here partially mitigated by the presence of flanking mirrors. In particular, it was found that reliable assessment of orolingual and forelimb dyskinesia from video recordings using the original scoring method was hampered by issues such as the fast movements exhibited by mice, challenges in obtaining 360° video coverage, and the video quality yielded by affordable recording hardware.

Importantly, a recent study by the Cenci laboratory, utilizing both live assessment and video recordings, described and pharmacologically validated a detailed method for scoring LID amplitude in mice (Sebastianutto et al., 2016). Furthermore, it was found that the frequency and amplitude dimensions of LID can be differentially responsive to drug treatments (Sebastianutto et al., 2016). The present studies were already ongoing at the time of this advancement. Future mouse studies, however, might be best served to adopt a scoring method which separately assesses both the frequency and amplitude of dyskinetic behaviors, such as the now available validated method of Sebastianutto et al. (2016), and to subject these dimensions to data analysis both separately and as a combined measure of dyskinesia.

The accuracy and validity of the present LID quantification method is an obvious methodological question. A number of findings suggest that the present method measures essentially the same phenomenon as previously described methods. Foremost, dyskinesia scores obtained with the present method and the original method of Lundblad et al. (2004) were found to be highly correlated. Note, however, that for MFB-lesioned animals only a truncated version of the original method could be used, as interruptibility was not measured. Findings in line with the previous literature included similar LID time courses as previously described (Lundblad et al., 2004; Francardo et al., 2011), more severe LID being exhibited by mice with near-total vs. partial nigrostriatal lesions (Lundblad et al., 2004; Francardo et al., 2011), alleviation of LID by nicotine in Study II (Huang, Grady, and Quik, 2011), and a very similar correlation between striatal BDNF levels and LID as was previously found between striatal *Bdnf* mRNA and LID in 6-OHDA-lesioned rats (Rylander et al., 2010).

A partial dose-response relationship between levodopa and dyskinesia scores was observed when data from several studies were combined (see Figure 15A). The lowest dose of levodopa (3 mg/kg) did not induce lower dyskinesia scores, preventing a conclusion of a full dose-response relationship. However, this may be explained by a number of methodological differences, including a different C57/BL6J substrain (α5-WT vs. RccHsd), younger mice (10–20 weeks vs. 28–30 weeks), the use of desipramine during lesioning, a different levodopa:benserazide ratio (1:5 vs. 4:1), and a longer levodopa treatment before assessment (3 vs. 2 weeks). High mean axial dyskinesia scores were induced by all doses in MFB-lesioned mice, and although consistently severe axial dyskinesia appeared a hallmark of the present MFB model, a lack of sensitivity in quantification cannot be entirely ruled out. Note that the separation of mild axial LID from levodopa-induced rotation (not considered dyskinesia) was at times difficult. Finally, pharmacological validation of the scoring method was not consistently achieved. While nicotine reduced LID in intrastriatally lesioned mice, the effects of nicotine on LID in MFB-lesioned mice were unclear (see 6.3.1.). Moreover, treatment with the known antidyskinetic drug amantadine failed to reduce LID scores in MFB-lesioned animals (see 6.3.2.). The readily apparent and entirely aberrant prolonging of dyskinesia by amantadine suggests, however, that the observed lack of efficacy was not due to a lack of sensitivity in quantification. In summary, available evidence suggests that the dyskinesia scores obtained using the present scoring method accurately reflect the time course and severity of mouse LID, although in hindsight some additional control experiments could have been performed. These could have included administration of amantadine to intrastriatally lesioned dyskinetic animals as well as direct comparisons of different levodopa doses.

Finally, statistical analysis of dyskinesia scores should be briefly discussed. As dyskinesia scoring scales used in the literature are mostly ordinal by nature, using parametric statistics (and, in fact, calculating such measures as sums or means) is in the strict sense inappropriate (Forrest and Andersen, 1986; Munzel and Langer, 2004). However, it is common and, argued by some statisticians, even fully acceptable practice to utilize parametric statistical analysis on ordinal scores or their summations (Forrest and Andersen, 1986; Munzel and Langer, 2004; Norman, 2010). Moreover, in the case of the original LID scoring method it has been argued that parametric analysis allows for remarkable descriptive power and is acceptable given that the scoring scale has some properties of a ratio scale and that its output is correlated with biochemical markers (Cenci and Lundblad, 2007). Parametric tests indeed allow more detailed analysis, particularly as a non-parametric test for analyzing two-way repeated measures data (a very common form of data in LID studies) is not readily available. As for the present scoring method, the linear correlation of LID with striatal BDNF arguably satisfies the latter condition. Nevertheless, as suggested by Cenci and Lundblad (2007), in the present thesis non-parametric tests were used in parallel with parametric tests. The results were generally along the same lines. However, in some cases group differences did not quite reach statistical significance with non-parametric analysis (see Figure 16). In other cases more complex effects, such as group differences appearing

only during some part of the experiment, could not be confirmed (see Figures 17–18). The latter is not surprising given the loss of information when integrating a data series into one value. In the end, the results of parametric statistical analysis were used as the primary results on which conclusions were based. This follows the large majority of previous studies by many laboratories which have only used parametric statistics in the analysis of data obtained with similar LID scoring methods (e.g., Santini et al., 2007; Buck and Ferger, 2008; Darmopil et al., 2009; Bido et al., 2011; Francardo et al., 2011; Thiele et al., 2011; Quik et al., 2012).

6.2. α5* nicotinic receptors in mouse models of Parkinson's disease

6.2.1. Attenuated dopaminergic neurodegeneration and motor dysfunction in mice lacking α5 nAChRs*

Nicotinic receptors containing the α5 subunit could have a role in Parkinson's disease and LID or their animal models, given the critical role of α5* nAChRs in the regulation of dopamine release in the dorsal striatum at least in mice (Salminen et al., 2004; Exley et al., 2012). To study this possibility, an extensive series of experiments utilizing α5 subunit-knockout mice was undertaken in order to characterize the role of α5* nAChRs in mouse models of PD and LID as well as the effects of the lack of α5* nAChRs on the dopaminergic system. These studies represent the first investigation of α5* nAChRs in the context of PD, and the findings of attenuated dopaminergic neurodegeneration and motor dysfunction in α5-KO mice raise intriguing possibilities for the use of α5 nAChRs as a drug target. Striatal markers of the dopaminergic system were unaffected, with the notable exception of reduced dopamine transporter function observed in α5-KO mice.

The primary finding was that the death of dopaminergic neurons induced by intrastriatal injections of 6-OHDA was attenuated in α5-KO mice, specifically within the medial cluster of the SNC. This neuroprotective effect was paralleled by attenuation of amphetamine-induced rotation, a widely-used test where rotational behavior relates to the degree of dopaminergic denervation (Iancu et al., 2005; Bové and Perier, 2012). The latter finding suggests that the neuroprotection was significant enough to be reflected in motor behavior. Importantly, no genotype difference in locomotor activity was found after amphetamine administration in intact animals, suggesting that the observed attenuation of rotational behavior was indeed linked to the lessened neurodegeneration. The present finding is also in line with a previous study, utilizing electrically lesioned rats, where damage to the medial but not the lateral substantia nigra was linked to amphetamine-induced ipsilateral rotational behavior (Vaccarino and Franklin, 1982). This further suggests that the less pronounced ipsilateral rotation observed in the present experiments was the result of attenuated neurodegeneration in the SNCM.

In female animals lesioned with intra-MFB 6-OHDA injections, lesion extent in the SNC did not differ between wild-type and α5-KO mice. Nevertheless, attenuated dopaminergic motor dysfunction was observed also in these α5-KO animals, as evidenced by a tendency for attenuated amphetamine-induced rotation as well as less severe LID. The contrasting SNC immunohistochemical results from the two experiments utilizing different lesion models could be explained by their different time courses. When utilizing the intrastriatal model, lesion extent was assessed 1–2 weeks after the rotametry experiments, capturing the state of the midbrain soon after the behavioral assays. In contrast, when utilizing the intra-MFB model, several months of chronic treatment with levodopa, benserazide, and either nicotine or vehicle interceded between the rotametry measurements and immunohistochemistry. Possible confounding phenomena include effects of levodopa, able to induce a TH-positive phenotype in at least striatal neurons (Darmopil et al., 2008; Francardo et al., 2011), or spontaneous neuronal recovery, reported in 6-OHDA-lesioned mice at least in the striatum (Bez et al., 2016). Alternatively, it is possible that the more severe dopaminergic neurotoxicity induced in the intra-MFB model resulted in the masking of any protective effect by α5-KO; note the suggested lesser suitability of the intra-MFB model for neuroprotection studies in general (Bové and Perier, 2012). In that case, the tendency for attenuated rotational behavior could suggest more efficient functional compensation in α5-KO mice. Finally, in a pilot experiment fewer successful intra-MFB lesions, as well as a tendency for attenuated amphetamine-induced rotation, were observed in male α5-KO mice. These findings further suggest attenuated dopaminergic neurodegeneration and motor dysfunction in mice lacking α5* nAChRs.

As mentioned, female α5-KO mice lesioned with intra-MFB 6-OHDA developed less severe LID. The attenuated LID could be explained by less severe denervation (Lundblad et al., 2004; Francardo et al., 2011), but no genotype difference in lesion extent was observed in these mice. Although the long period of chronic drug treatments may explain the lack of a difference in lesion extent (see above), the attenuated dyskinesia in α5-KO animals may also have been the result of some other mechanism. This mechanism could be analogous to similar findings in mice lacking the α6 nAChR subunit (Quik et al., 2012), and the finding suggests the involvement of α5* nAChRs in LID pathophysiology. The important role of α5* nAChRs in the regulation of striatal dopamine release (Salminen et al., 2004; Exley et al., 2012) and the decreased striatal DAT function in α5-KO mice suggest at first glance that the attenuated LID could be the result of altered release and/or uptake of levodopa-derived dopamine. However, given the near-total loss of striatal dopaminergic innervation observed in MFB-lesioned mice in the present studies, these explanations are unlikely. Other α5* nAChR populations that could be involved include those regulating serotonergic neurotransmission in the dorsal raphe nucleus (Besson et al., 2016), given the importance of raphestriatal serotonergic projections for LID (see 2.3.2.), or those modulating striatal GABA release (McClure-Begley et al., 2009). α5* nAChR disruption with e.g., selective antagonists or agonists (*via* desensitization) could thus represent a novel treatment avenue

for LID. See also below (6.3.1.) for related discussion on the effects of nicotine on LID in α5-KO mice.

Interestingly, while dopaminergic cell loss in the SNCD was more severe in the intra-MFB model than in the intrastriatal model (as expected), cell loss in the SNCM was in fact less severe in the intra-MFB model. This suggests that spontaneous or drug-induced recovery may indeed have occurred and perhaps been more pronounced within the SNCM. Alternatively, the relatively well-spared SNCM could be due to a presence of projections from the SNCM to the dorsal striatum that do not pass through the MFB coordinates where 6-OHDA was injected. In the rat, projections of the SNC exhibit a relatively distinct topographic segregation, with medial parts projecting to the dorsomedial striatum (and partly to the ventral striatum) and lateral parts projecting to the dorsolateral striatum (Joel and Weiner, 2000). This suggests that there may also exist anatomical differences in the projections of the SNCM and the SNCD in mice (note that varying terminology across and even within species complicates comparisons; see e.g., Fu et al., 2012). In the intrastriatal model, the 6-OHDA injections were targeted approximately at the center of the striatum on the lateromedial axis and resulted in similar lesions in the SNCD and the SNCM. Finally, chronic nicotine treatment had no effect on the lesion extent of MFB-lesioned animals. This finding is in line with a previous study showing no neurorestoration by post-lesion chronic nicotine treatment in 6-OHDA-lesioned rats or MPTP-lesioned primates (Huang et al., 2009).

Surprisingly, male mice lesioned with intrastriatal 6-OHDA injections exhibited a contrasting pattern of genotype differences in rotational behavior. As no sex or genotype differences were found in intact animals' motor response to amphetamine, and in the intra-MFB model both male and female α5-KO mice showed a tendency for attenuation of amphetamine-induced rotation, this sex difference appears to be linked specifically to the intrastriatal 6-OHDA model. While little is known about possible sex differences related to α5* nAChRs, the female sex hormone progesterone has been shown to upregulate α5* expression (Gangitano et al., 2009). The lack of significant attenuation of rotational behavior in response to α5 deletion in male mice could therefore have been the result of lower α5* nAChR expression already in wild-type animals. The lack of a similar sex difference in the intra-MFB model could, then, be related to the near-total loss of striatal α5-expressing dopamine terminals. The contrasting behavioral findings in male mice could also be related to the sex differences observed in some biomarkers of the dopaminergic system. More efficient dopamine uptake in male mice could explain the shorter duration of amphetamine's effects seen in intrastriatally lesioned male animals, although no such sex difference was observed in intact or MFB-lesioned animals. Furthermore, a relatively larger lesion-induced reduction in D1 and possibly D2 receptor mRNA was observed in the intrastriatally lesioned male animals when compared to female animals. A greater decrease in dopamine receptors in male animals could result in greater sensitization of the remaining receptors and thus affect in particular the effects of the direct dopamine agonist

apomorphine. However, the explanation for the more pronounced apomorphine-induced rotation observed specifically in male α5-KO mice remains unclear.

6.2.2. Potential mechanisms and implications of attenuated neurodegeneration

The dopaminergic neuroprotection observed in α5-KO animals may be linked to their reduced striatal DAT function. 6-OHDA is thought to enter dopaminergic neurons *in vivo* through the DAT (Blum et al., 2001), and the reduced DAT function may thus have led to attenuated neurotoxicity. Supporting this hypothesis are previous findings in rats, linking differences in DAT protein levels to the differential vulnerability of midbrain dopaminergic neuron populations to 6-OHDA (González-Hernández et al., 2004). Notably, acute nAChR activation has previously been found to increase dopamine clearance in the striatum and DAT cell surface expression in the PFC (Middleton et al., 2004; Zhu et al., 2009), showing that nAChR signaling can indeed modulate DAT function. As no genotype difference in striatal DAT protein levels was found in the present study, the reduced dopamine uptake observed in α5-KO animals seems to be mainly the result of reduced DAT activity. DAT activity and membrane expression is regulated by phosphorylation (Vaughan et al., 1997; Morón et al., 2003), and the phosphorylation site T53 has a major role in activity modulation (Foster et al., 2012). However, no genotype difference in striatal phospho (T53)-DAT levels was found, suggesting that the decreased activity was mediated by other mechanisms. Finally, DAT measurements were performed only in the striatum. Thus, it remains an open question whether also the function of the dopamine transporters localized on axon fibers in the MFB (Ciliax et al., 1995) was reduced and whether this might in part explain the lack of neuroprotection in the intra-MFB model.

It should be noted, however, that a number of *in vitro* studies have not been able to demonstrate the DAT-dependence of 6-OHDA cytotoxicity (Blum et al., 2001; Storch et al., 2004). Another explanation for the attenuated dopaminergic denervation in animals lacking α5* nAChRs could be reduced calcium influx and consequently reduced oxidative stress. Incorporation of the α5 subunit into a nAChR results in increased calcium permeability (Tapia et al., 2007), and α5* nAChRs have an essential role in nAChR-mediated calcium fluxes in at least some dopaminergic neurons of the ventral midbrain (Sciaccaluga et al., 2015). On the other hand, the neurotoxic effects of 6-OHDA are suggested to be caused by oxidative stress and amplified by cytoplasmic free calcium (Blum et al., 2001). Supporting this suggestion is a finding of increased striatal intracellular calcium in 6-OHDA-lesioned rats (Kumar et al., 1995). Interestingly, cytosolic reactive oxygen species are able to inactivate nAChRs, possibly as a protective mechanism against excess calcium influx (Campanucci et al., 2008; Krishnaswamy and Cooper, 2012). Conversely, this suggests that calcium influx through nAChRs can indeed be a significant cause of oxidative stress and, thus, that its dampening as a result of a lack of α5* nAChRs could have protective consequences.

It is unclear why a neuroprotective effect mediated by either reduced DAT function or reduced calcium influx would manifest specifically in neurons of the SNCM. Different SN subdivisions express dopaminergic neurons of various phenotypes; it may be of relevance that when compared to dopaminergic neurons of SNCD, a much higher proportion of dopaminergic neurons in the mouse SNCM express the calcium-binding protein calbindin (Fu et al., 2012), suggesting that they may be more resistant to calcium-linked toxicity. Indeed, low calbindin expression has previously been suggested as one explanation for the selective vulnerability of SNC dopaminergic neurons in PD (Brichta and Greengard, 2014). On the other hand, no difference in DAT expression in dopaminergic neurons was found between mouse SNCD and SNCM (Fu et al., 2012). It remains to be determined whether dopaminergic neurons of the different mouse SNC regions differ in other aspects of calcium signaling or their expression of α5* nAChRs. Note that while the lateral SNC is more affected than the medial SNC in human PD, both are significantly degenerated (Fearnley and Lees, 1991). It is difficult, however, to draw any conclusions on the significance of the SNCM-specificity of the neuroprotection for human PD, given marked differences in nigrostriatal anatomy between rodents and primates (Joel and Weiner, 2000).

It is notable that calcium-linked oxidative stress in particular, but also the DAT, have been suggested to be of major importance in the pathophysiology of human PD. Oxidative stress caused by disturbances in intracellular calcium homeostasis has been suggested as an important inducer of mitochondrial dysfunction in PD, and the selective vulnerability of nigrostriatal dopaminergic neurons has been linked for example to their reliance on membrane L-type calcium channels for autonomic pacemaking (Brichta and Greengard, 2014; Michel et al., 2016). It is quite conceivable that the highly calcium-permeable α5* nAChRs could act as one mediator of calcium overload in PD pathogenesis. On the other hand, also DAT expression has been suggested to play a role in selective vulnerability. DAT expression has been linked to the sensitivity of dopaminergic neurons to various neurotoxins in animal models, and also suggested to account for the region-specificity of dopaminergic denervation in human PD (González-Hernández et al., 2004; Storch et al., 2004). Thus, previous literature supports an argument that the neuroprotection resulting from the lack of α5* nAChRs in mice could have clinical significance for the human disease. As such, the present results could represent a significant finding in the field of dopaminergic neuroprotection, suggesting potential for α5* nAChR disruption as a novel avenue for the treatment of PD. Further studies are needed to more clearly elucidate the mechanisms of the attenuated denervation and motor dysfunction. Such studies could for example investigate the effects of α5 gene deletion in genetic mouse models of Parkinson's disease or on other measures of parkinsonism than drug-induced rotational behavior. Further studies could also investigate the contribution of α5* nAChRs to the neuroprotection and more efficient functional compensation observed in animals treated chronically with nAChR agonists (see 2.4.4.). Agonist-induced chronic desensitization and genetic deletion of α5* nAChRs could conceivably have partially similar outcomes, resulting in neuroprotection and attenuated motor dysfunction.

On the other hand, the possibility that the observed neuroprotective effect is specific to the 6-OHDA model must also be acknowledged. This may be true particularly if the attenuated neurodegeneration was indeed the result of reduced DAT function and diminished 6-OHDA uptake. A potentially highly relevant question that follows is whether alterations of DAT function have been ruled out in previous studies on nAChR-mediated neuroprotection utilizing 6-OHDA models. Moreover, also MPTP exerts its neurotoxicity *via the DAT* (Storch et al., 2004). Given that DAT function can be affected both by nAChR activation (Middleton et al., 2004; Zhu et al., 2009) and by changes in nAChR subtype distribution (present findings), an essential control experiment could be missing from much of the literature. It is consoling to note, however, that at least one study has found DAT expression and function in the mouse striatum to be mostly resistant to chronic nicotine treatment (Marks et al., 2014). The neuroprotective effects of smoking have also been extensively demonstrated in humans (see 2.4.4.), showing that nAChR-mediated neuroprotection as a whole is unlikely to be an artefact. Nevertheless, this intriguing and somewhat alarming question deserves further study.

Another important question regarding the potential clinical implications of the present findings is whether human nigrostriatal neurons express α5* nAChRs. α5 subunit mRNA was detected in the monkey SNC (Han et al., 2000) but only weakly in most human brain areas, including the SN and the caudate-putamen (Flora et al., 2000). Furthermore, in immunoprecipitation experiments no α5 subunit protein was reliably detected in the monkey or human striatum (Quik et al., 2005; Gotti et al., 2006). Thus, the present findings could be applicable only to rodents. However, it is possible that the α5 antibodies used in the mentioned immunoprecipitation experiments were not able to detect some (or any) portion of human α5* nAChRs. Advances in antibody design might hopefully enable the conducting of additional studies on this issue. Furthermore, the subunit composition of nAChRs of the human SNC remains unknown (Zoli et al., 2015), leaving open the possibility that α5* nAChRs could be expressed somatodendritically. Finally, the links between *CHRNA5* gene variants and nicotine addiction (Bierut et al., 2008; Hung et al., 2008; Thorgeirsson et al., 2008; Weiss et al., 2008) suggest that α5* nAChRs do have an important role at least within the mesolimbic dopaminergic pathway of humans.

6.3. Nicotinic receptors in mouse models of levodopa-induced dyskinesia

6.3.1. Alleviation of moderate but not severe LID by chronic nicotine treatment

The second main research avenue of the present studies was the further investigation of the antidyskinetic effects of long-term nicotine treatment, previously described in a number of animal models of LID by Quik and colleagues (see 2.4.4.). Data from Study II suggest that chronic nicotine treatment in drinking water inhibited the development of LID in mice

suffering from partial dopaminergic denervation. In addition, data from synaptosomal [^{3}H]dopamine release experiments suggest that the the antidyskinetic effects may have been mediated by downregulation of striatal presynaptic α6β2* nAChRs (see 6.3.3.). The results are in line with the previous preclinical findings and represent the first independent replication of the antidyskinetic potential of nicotine published in peer-reviewed form. Thus, the present findings give additional support to the hypothesis that long-term nicotine treatment may have beneficial effects on dyskinesia associated with the treatment of Parkinson's disease with levodopa. The results of a recently finished clinical trial studying the effects of oral nicotine on LID (Lieberman et al., 2018) should, when published, shed more light on the implications of these preclinical findings for human PD.

An important question when considering dyskinesia-alleviating effects is whether these effects reflect a reduction in LID specifically. A treatment that reduces the effects of levodopa generally would inhibit LID but be undesirable due to the simultaneous inhibition of beneficial antiparkinsonian effects. In the present studies, the effects of the nicotine treatment on the antiparkinsonian efficacy of levodopa were not investigated. Note, however, that a similar nicotine treatment in drinking water increased locomotion and striatal dopamine release in mice (Gäddnäs et al., 2001), suggesting that at least a general motor-depressant effect can be ruled out. Moreover, Quik and colleagues have routinely investigated parkinsonism and levodopa's antiparkinsonian effects in their extensive studies, and have reported no change by nAChR agonist treatment in any animal model. This suggests that the LID alleviation seen in nicotine-treated animals is not the result of generally reduced levodopa efficacy.

In subsequent experiments, the effects of chronic nicotine treatment in drinking water were studied in mice of the intra-MFB model suffering from near-total dopaminergic denervation (Studies I and III). While statistical analyses of the data show that the nicotine treatments did affect LID, unlike in Study II clear antidyskinetic effects and lower dyskinesia scores than in control animals were not observed. On the contrary, in Study III LID may have been transiently exacerbated by the nicotine treatment. In Study I the nicotine treatment may have reduced LID over time in wild-type animals, but other interpretations of the data are also possible.

An arguably very plausible explanation for the lack of similarly robust antidyskinetic efficacy by nicotine in MFB-lesioned mice is the much more extensive dopaminergic lesion. The extent of the lesion may be particularly important in determining treatment effectiveness in the case of drugs (such as nicotine) which affect the presynaptic receptors of striatal dopamine terminals, as in a severe lesion these receptors are mostly lost. The finding of no clear LID alleviation in near-totally lesioned mice is partially in line with previous rodent and monkey studies, where the most severely lesioned animals were typically found to be less responsive or even completely unresponsive to antidyskinetic nicotine treatment (Huang, Campos, et al., 2011; Huang, Grady, and Quik, 2011; Quik, Campos, and Grady,

2013; Quik, Mallela, Chin, McIntosh, et al., 2013). These findings suggest that a partially intact nigrostriatal pathway may be required or at least highly conducive for the treatment of LID with nicotine. The potential treatment of LID with nAChR agonists in PD patients could thus lose effectiveness as the disease progresses.

On the other hand, the present findings are also partially in contrast with previous studies, where nicotine treatment in drinking water did inhibit LID in severely lesioned mice and rats with as little as less than 1 % of striatal dopaminergic innervation remaining (Huang, Campos, et al., 2011; Huang, Grady, and Quik, 2011; Quik et al., 2012; Quik, Campos, Bordia, et al., 2013; Quik, Campos, and Grady, 2013). The reasons for this discrepancy are unclear, but note that the methods of lesioning, LID induction, and chronic nicotine treatment were similar to the previous mouse studies (Huang, Grady, and Quik, 2011; Quik et al., 2012; Quik, Campos, and Grady, 2013). Although nicotine intake was not measured on the individual level, average nicotine intake was very similar in both the intrastriatally lesioned mice, where an antidyskinetic effect was observed, and in the MFB-lesioned mice. Moreover, plasma and brain nicotine concentrations during administration in drinking water were determined in previous studies at the research site (Pekonen et al., 1993; Pietilä et al., 1995; Gäddnäs et al., 2001), and found to reach similar or higher levels than those reported in heavy smokers, sufficient to induce the nAChR desensitization that is thought to underlie LID alleviation (plasma 33–144 ng/ml, brain 243–329 ng/g, depending on the time of day). Also note that the sex and exact age of the mice were not reported in the previous studies, making comparison on that basis impossible (Huang, Grady, and Quik, 2011; Quik et al., 2012; Quik, Campos, and Grady, 2013).

The timing of antidyskinetic drug administration could also affect its efficacy. Here, attenuation of LID by nicotine was observed when the chronic drug treatments were initiated simultaneously, while no clear antidyskinetic effects were observed when treating pre-established LID with nicotine. Previous mouse studies have reported robust antidyskinetic effects also on pre-established LID (Huang, Grady, and Quik, 2011; Quik et al., 2012; Quik, Campos, and Grady, 2013). However, the dose of levodopa used in the present Study III (6 mg/kg) was higher than in most of the previous studies, where dyskinesia induced by 6 mg/kg levodopa was studied (and found to be alleviated by nicotine) only using nicotine pretreatment (Huang, Grady, and Quik, 2011). In addition, at least axial LID may have been in general more severe in the present studies utilizing MFB lesions than than in those of Quik and colleagues. Observations indirectly suggesting this include continuous axial dyskinesia replacing most normal behaviors, which would certainly have prevented the use of some methods successfully used in the previous studies, primarily the forelimb use asymmetry test (Huang, Grady, and Quik, 2011). Direct comparison of LID severity is difficult, however, due to the previous studies utilizing their own modification of the LID scoring method (Huang, Grady, and Quik, 2011). All in all, it is possible that particularly in Study III the combination of a near-total lesion, high levodopa dose, and pre-existing sensitization to levodopa resulted in dyskinesia that was too severe and ingrained to yield

to attenuation by the nicotine treatment. This situation could be comparable to starting nicotine treatment in advanced PD associated with a high levodopa dose regime and severe LID. Thus, it may turn out to be necessary to begin potential clinical antidyskinetic treatments with nAChR agonists as early as possible, perhaps even before significant LID has developed.

A notable difference between the various present studies was that desipramine pretreatment was used to inhibit noradrenergic neurodegeneration only in the studies where clear (Study II) or possible (Study I) attenuation of LID was observed. In rats lesioned with intra-MFB 6-OHDA injections, desipramine pretreatment has resulted in both decreased and increased LID (Fulceri et al., 2007; Barnum et al., 2012). Thus, it is unclear what role the desipramine pretreatment may have had in the expression of LID in the present studies. However, as none of the previous rodent studies on antidyskinetic effects of nAChR agonists used desipramine (Bordia et al., 2008; Huang, Campos, et al., 2011; Huang, Grady, and Quik, 2011; Quik et al., 2012; Quik, Campos, and Grady, 2013), it is unlikely that the lack of desipramine pretreatment would fully explain the lack of efficacy of nicotine in Study III.

A final potential explanation for the partially differing results between the present studies utilizing MFB-lesioned mice and the studies of Quik et al. is the use of a different LID scoring method. As different dimensions of LID can be differentially responsive to drug treatment (Sebastianutto et al., 2016), different scoring methods could conceivably result in different apparent treatment outcomes. For instance, nicotine might decrease the frequency but not amplitude of dyskinesia in severely dyskinetic mice. To investigate this possibility, in Study III the number of instances of severe axial dyskinesia per minute was adopted as a frequency measurement to complement the mainly amplitude-based scoring criteria for axial LID. However, nicotine had no effect on this measure, suggesting that this potential explanation is also unlikely to account for the negative results.

Notwithstanding the above discussion, it should be stressed that statistical analyses show that the nicotine treatments did affect LID also in MFB-lesioned mice. Whether these effects were alleviating or not, an interesting question is the identity of the nAChR populations that mediate presumably dopamine-related motor effects in conditions of near-total nigrostriatal denervation. Note, again, that such effects were observed not only in the present studies but in a number of previous rodent studies (Huang, Campos, et al., 2011; Huang, Grady, and Quik, 2011; Quik, Campos, Bordia, et al., 2013). Thus, it appears reasonably certain that other nAChR populations than those expressed on nigrostriatal neurons of the lesioned hemisphere also contribute to the modulation of LID by nAChR ligands. The identity of these receptor populations has not so far been directly investigated, but they could be potential drug targets in the treatment of LID associated with advanced PD. As there is no evidence of nAChRs on striatal MSNs (Quik and Wonnacott, 2011), potentially involved receptor populations could include presynaptic nAChRs expressed on striatal serotonin or glutamate terminals (Schwartz et al., 1984; Kaiser and Wonnacott,

2000; Reuben and Clarke, 2000), both of which are implicated in LID pathophysiology (see 2.3.2.). As serotonergic terminals are particularly critical for LID, being responsible for the non-physiological release of levodopa-derived dopamine, it is unfortunate that very little is known about their presynaptic nAChRs. Moreover, any of the diverse nAChR populations that are expressed widely across the entire brain could in principle contribute. Some drug effects might conceivably also be mediated by nigrostriatal nAChRs of the intact brain hemisphere, even though LID in the rodent model is predominantly expressed on the contralateral side that is controlled by the lesioned hemisphere.

Finally, the contribution of α5* nAChRs to nicotine's effects on LID was investigated in Study I. The chronic nicotine treatment had differing effects on LID in wild-type and α5-KO mice; more specifically, LID appeared to be attenuated over time in wild-type but not α5-KO mice, although statistical analyses did not definitively confirm this particular interpretation. Nevertheless, the genotype difference suggests that α5* nAChRs are involved in the modulation of LID by nicotine. This would not be entirely surprising, given the finding of attenuated LID in α5-KO mice that suggests the involvement of α5* nAChRs in LID pathophysiology (see above). Thus, the α4β2* nAChRs shown to mediate nicotine's effects on LID in mice (Huang, Grady, and Quik, 2011; Quik, Campos, and Grady, 2013) could very well include α4α5β2 nAChRs. Again, the presence of a significant effect in near-totally lesioned animals suggests the involvement of other nAChRs than those on dopaminergic neurons. α5-containing nAChRs exhibit altered desensitization kinetics (Tapia et al., 2007; Grady et al., 2012), suggesting that the manipulation of α5* nAChRs could have particularly significant effects on the postulated desensitization-based effects of nicotine on LID (Bordia et al., 2010; Bordia et al., 2016). α5* nAChRs could, as already proposed above, thus be a potential target for more selective antidyskinetic treatments. However, additional studies more clearly demonstrating the nature of the α5*-mediated pharmacological effects on LID are necessary.

6.3.2. No alleviation of severe LID by α7 nAChR agonist and amantadine treatments

In Study III, chronic administration of the selective α7 nAChR partial agonist AZD0328 had no effect on pre-established LID in MFB-lesioned mice. While α7 nAChRs are not expressed on striatal dopamine terminals (Salminen et al., 2004), somatodendritic α7 nAChRs modulate the activity of SNC dopaminergic neurons in rodents (Klink et al., 2001). Thus, the near-total nigrostriatal denervation could have eliminated potential sites of action also in this case. While in primates beneficial effects of α7 agonists on LID have been reported also in severely lesioned animals, the loss of striatal innervation was not total (Zhang et al., 2015). A too short treatment time or too low dosages are unlikely explanations for the lack of efficacy. Monkey studies have shown significant effects by α7 agonists already within 1–2 weeks (Di Paolo et al., 2014; Zhang, McGregor, et al., 2014; Zhang et al., 2015), and comparable AZD0328 doses were shown in mice to result in various behavioral effects, changes in dopamine release, and (at the highest dose) maximal brain α7

receptor occupancy (Sydserff et al., 2009; Werkheiser et al., 2011). It is, again, also possible that the pre-established LID exhibited in the present studies was in general too severe for alleviation by nAChR agonists. However, as no previous studies on selective α7 nAChR agonists in dyskinetic rodents have been published, it may also be that α7 agonism is not antidyskinetic in the mouse. Although α7-knockout mice exhibit more severe dyskinesia, they respond to antidyskinetic nicotine treatment similarly or even better than wild-type mice (Quik, Campos, and Grady, 2013). Thus, in mice α7 agonism does not appear to have a major role in nicotine's antidyskinetic effect either. Note also that a recent clinical study observed no reduction in LID by α7 nAChR agonist treatment (Trenkwalder et al., 2016).

The effects of acute treatments with amantadine on LID were investigated in an additional study. Surprisingly, the treatment of MFB-lesioned mice with amantadine failed to decrease LID severity. This finding is in contrast to previous mouse studies, which have reported robust reductions of pre-established LID using similar amantadine and levodopa dosages and timing of administration (Lundblad et al., 2005; Bido et al., 2011; Sebastianutto et al., 2016). Moreover, amantadine consistently prolonged LID duration in the present study, an observation not reported previously. The prolonging of LID was clear and readily apparent at the time of the experiments. Moreover, lower doses of amantadine did not have as clear LID-prolonging effects, suggesting a dose-response relationship. While amantadine's targets are numerous and its mechanisms of action are not completely understood (Bido et al., 2011), interestingly it is an antagonist of both α7 and β2* nAChRs (Albuquerque et al., 1998). Thus, the antidyskinetic effects of amantadine could conceivably be mediated by nAChRs on dopaminergic neurons and be lost in conditions of near-total dopaminergic denervation. On the other hand, a previous study reported effective alleviation of LID by amantadine also in MFB-lesioned mice, although biochemical characterization of the lesions was not carried out (Sebastianutto et al., 2016). All in all, the unusual behavioral effects of the known antidyskinetic drug amantadine suggest that the unresponsiveness to antidyskinetic drug treatment observed in the severely dyskinetic MFB-lesioned mice may have been more general instead of specific to nAChR agonists. Care should be taken in future studies to investigate potential antidyskinetic drugs using different severity stages of dopaminergic denervation and LID. Effects may be blunted or even paradoxical in severe conditions, and treatments potentially useful in earlier disease could be unnecessarily discarded.

6.3.3. *Effects of antidyskinetic nicotine treatment on striatal dopamine release*

The effects of 6-OHDA lesioning and chronic nicotine treatment on striatal dopamine release were investigated in Study II by measuring both basal and nicotine-stimulated release of [^{3}H]dopamine from synaptosomes. α6β2* and α4(non-α6)β2* nAChRs may differentially regulate dopamine release in the dorsal and ventral striatum (Exley et al., 2012), and are differentially affected by nigrostriatal denervation (Bordia et al., 2007)

and chronic nicotine treatment (Lai et al., 2005; Marks et al., 2014). Therefore, the dorsal and ventral striatum were studied separately, and α6β2*-mediated and α4(non-α6)β2*-mediated release was separated using CtxMII.

Interestingly, no difference was found in the proportions of [^{3}H]dopamine release mediated by α6β2* versus α4(non-α6)β2* nAChRs, in contrast to voltammetry studies in mouse brain slices (Exley et al., 2012) but in line with previous synaptosomal release studies (Grady et al., 2002; Salminen et al., 2007). Note that the synaptosome technique uses continuous superfusion that is thought to largely abolish indirect effects mediated by the release of other neurotransmitters. These studies thus strongly suggest that such indirect effects significantly contribute to nAChR-mediated regulation of dopamine release. The nigrostriatal 6-OHDA lesion had no effect on nAChR-mediated [^{3}H]dopamine release when normalized to basal release, suggesting that despite the significant loss of dopamine terminals the remaining presynaptic nAChRs functioned with normal efficiency. Non-normalized basal release was reduced by the lesion, as expected. The lesion also did not change the relative contribution of α6β2* and α4(non-α6)β2* nAChRs to [^{3}H]dopamine release, largely in line with a previous voltammetric study in rats (Perez et al., 2010). Ligand binding studies have shown a preferential loss of α6β2* nAChRs after dopaminergic denervation (Bordia et al., 2007; Perez et al., 2010), but this likely reflects their preferential expression on dopamine terminals in the striatum.

In animals treated chronically with levodopa and either nicotine or vehicle, a partial recovery of basal [^{3}H]dopamine release in the lesioned dorsal striatum was observed irrespective of treatment group, possibly representing spontaneous recovery as reported in some previous mouse studies (Stanic et al., 2003; Bez et al., 2016). Note, however, possible confounding effects by the different sex distribution in the related experimental groups. Intriguingly, the chronic nicotine treatment appeared to restore basal [^{3}H]dopamine release in the lesioned ventral striatum up to intact levels. Although neurorestoration by post-lesion nicotine treatment has been observed in some studies (Janson, Fuxe, Agnati, et al., 1988; Fuxe et al., 1990; Janson and Møller, 1993), most studies reporting neuroprotective effects have included a pretreatment period (see Table 2), and a study directly comparing pre- and post-treatments found no neurorestoration (Huang et al., 2009). Thus, the present result may represent functional compensation rather than a regrowth of dopamine terminals.

While the chronic nicotine treatment had no effect on α4(non-α6)β2*-mediated [^{3}H] dopamine release, α6β2*-mediated release was significantly reduced in nicotine-treated animals in the lesioned dorsal striatum and in both the intact and lesioned ventral striatum. The reduction in α6β2*-mediated release could reflect downregulation and/or desensitization. A number of previous findings in mice suggest that downregulation is the more likely explanation. Previous synaptosome studies utilizing the same technique suggest that nAChRs would have mostly recovered from any acute desensitization by the time of measurement (Grady et al., 1994; Grady et al., 2012). Furthermore, previous studies have

reported α6β2* downregulation in the mouse striatum after chronic nicotine treatment (Lai et al., 2005; Marks et al., 2014). Moreover, antidyskinetic chronic nicotine treatment was associated with α6β2* downregulation also in rats (Bordia et al., 2010; Bordia et al., 2013). Although in the former study this was observed only in the unlesioned hemisphere, α6β2* nAChR levels of the lesioned hemisphere were so low that differences in expression may have been impossible to detect. Note also that in these studies α4(non-α6)β2* nAChRs were upregulated or unchanged and in general found to be less sensitive to regulation, in line with the present results (Lai et al., 2005; Bordia et al., 2010; Bordia et al., 2013; Marks et al., 2014).

The present finding of reduced α6β2*-mediated release is also in line with further rodent findings suggesting that α6* nAChRs have a significant role in the antidyskinetic effects of nicotine (Quik et al., 2012) and that the effects are mediated by downregulation or desensitization of striatal presynaptic nAChRs and dampening of dopaminergic activity (Bordia et al., 2010; Bordia et al., 2013). Thus, the present results further support a role for α6β2* nAChRs as an important mediator of dyskinesia-alleviating effects of nAChR agonists and as a possible drug target in the treatment of LID. Notably, within the basal ganglia α6β2* nAChRs are selectively expressed on dopaminergic neurons across species (Quik, Sum, et al., 2003; Quik et al., 2005; Gotti et al., 2006), suggesting that they may hold promise for more selective targeting than can be achieved with non-selective drugs such as nicotine. However, selective targeting of specifically striatal α6* nAChRs could be complicated by their expression also outside of the basal ganglia (Le Novere et al., 1996). Furthermore, the expression of α3/α6* nAChRs is more widespread in the human than the rodent or monkey brain (Quik et al., 2004; Bohr et al., 2005), although it is unclear what proportion of those consist specifically of α6* nAChRs.

In contrast to the present study, a previous study in rats found that antidyskinetic nicotine treatment was associated with a decrease also in α4(non-α6)β2*-mediated [^{3}H] dopamine release from striatal synaptosomes (Bordia et al., 2013). The explanation for the discrepancy is unclear, but could be the result of a different species or a different lesion model (intrastriatal vs. intra-MFB 6-OHDA). Note also that unlike in the previous study, in the present study nicotine-stimulated dopamine release was normalized to basal release to investigate the functioning of remaining nAChRs independently of the release-reducing effects of the lesion. The previous result of decreased α4(non-α6)β2*-mediated dopamine release is also difficult to reconcile with the simultaneously observed upregulation of the same nAChR subtype (Bordia et al., 2013), perhaps suggesting that upregulation could there have selectively affected nAChRs not located on dopamine terminals or that desensitization kinetics in the synaptosome assay (see above) could be different in rats than in mice.

Studies in α4-KO mice do suggest that nicotine's antidyskinetic effects are mediated also by α4* nAChRs (Quik, Campos, and Grady, 2013). Presynaptic α4α6β2β3 nAChRs are particularly sensitive to nicotine (Salminen et al., 2007), are suggested to be important

mediators of the antidyskinetic effects (Quik, Campos, and Grady, 2013), and are blocked by CtxMII. The loss of antidyskinetic effects by nicotine in α4-KO mice could thus be explained by the loss of this specific nAChR subtype, while its downregulation would not affect α4(non-α6)β2*-mediated [^{3}H]dopamine release. Both the present and the previous results in mice are therefore fully compatible with the hypothesis of α4α6β2β3 nAChRs as a critical mediator of LID alleviation. Alternatively, it should be noted that α4(non-α6)β2* nAChRs are the most widely expressed nAChR subtype (Millar and Gotti, 2009). It is thus very possible that other α4* nAChR populations and/or mechanisms than those related to striatal dopamine release would contribute to the antidyskinetic effects of nicotine – particularly when again considering the antidyskinetic effects previously observed in rodents suffering from practically total nigrostriatal denervation (see 6.3.1. above).

6.3.4. Effects of levodopa and nicotine treatments on BDNF

LID is associated with aberrant synaptic plasticity (Picconi et al., 2003; Thiele et al., 2014; see 2.3.2.). The BDNF, as an important regulator of plasticity (Bramham and Messaoudi, 2005), may be involved. In particular, levodopa treatment has been suggested to result in elevated BDNF in corticostriatal neurons and increased striatal BDNF release (Guillin et al., 2001). The increased striatal BDNF levels may then lead to such phenomena as D3R overexpression (Guillin et al., 2001) or serotonergic hyperinnervation (Rylander et al., 2010; Tronci et al., 2017). To further investigate the association of BDNF and LID, BDNF protein levels were measured from the striatum and the PFC of the mice of Study III that were treated chronically with levodopa and either nicotine or vehicle.

BDNF protein levels in the lesioned striatum and LID severity were positively correlated, similar to a previously reported correlation between striatal *Bdnf* mRNA and LID severity in rats (Rylander et al., 2010). The observed correlation further supports a role for BDNF in LID pathophysiology, demonstrating that the previously reported mRNA level correlation is present also when measuring the protein, a relationship which is not always predictable in the case of BDNF (McAllister et al., 1999). However, no differences in BDNF protein levels between the intact and lesioned hemispheres were observed, in contrast to earlier findings on *Bdnf* mRNA in rats (Guillin et al., 2001; Rylander et al., 2010). Thus, the present findings do not directly support the hypothesis of Guillin et al. (2001) that LID is caused by a levodopa-induced elevation of corticostriatal BDNF that is further enhanced in conditions of dopaminergic denervation. However, the contrasting results could be explained by the considerably longer duration of levodopa treatment (months vs. days/weeks). The elevated corticostriatal BDNF could be related to the early phase of sensitization to levodopa in the lesioned hemisphere and thus be undetectable after a longer treatment. Note also that while striatal BDNF protein is mainly derived from corticostriatal glutamatergic afferents, nigrostriatal dopamine terminals do represent a minor source of striatal BDNF (Altar et al., 1997). Unlike in the present study, a near-total depletion of striatal dopamine in 6-OHDA-lesioned rats was associated with a modest (14 %) decrease in striatal BDNF protein levels

(Altar et al., 1997). While the reason for this discrepancy is unclear, opposite changes in striatal BDNF by the nigrostriatal lesion and the levodopa treatment may in principle have occurred.

In the present study, chronic nicotine treatment had no effect on striatal BDNF levels, in line with a previous study on long-term nicotine administration in drinking water (Kivinummi et al., 2011). Other studies have, however, reported both increases and decreases in striatal BDNF after chronic nicotine administration via various routes (Yeom et al., 2005; Correll et al., 2009; Ortega et al., 2013). In the present study, the nicotine treatment significantly decreased BDNF levels in the PFC, similarly as in a previous study on rat frontal cortex utilizing repeated nicotine injections (Brown et al., 2006). While the previous study suggested that the nicotine-induced BDNF decrease was related to a withdrawal stress response, the present study included no withdrawal period, making this explanation unlikely. Note also a contrasting report of a nicotine-induced increase in cortical BDNF in another rat study (Czubak et al., 2009). All in all, effects of chronic nicotine treatment on BDNF appear highly variable. It is also unclear whether the nicotine-induced decrease in BDNF levels in the PFC was related to the changes in LID observed in the present studies. Mice in each study were treated with similar nicotine administration regimes and showed a similar average daily nicotine intake, but as the effects on LID varied from study to study, clear conclusions cannot be drawn.

Finally, it should be noted that contrary to rodent studies, in primates lesioned bilaterally with MPTP no difference in striatal BDNF protein levels between dyskinetic and non-dyskinetic animals was found after four weeks of levodopa treatment, and striatal BDNF protein levels were decreased when compared to saline-treated animals (Samadi et al., 2010). Similarly, PD patients carrying a genetic polymorphism associated with lower activity-dependent secretion of BDNF were found to be at higher risk of early development of LID (Foltynie et al., 2009; Kusters et al., 2018). In primates, it could thus in fact be decreased levels of BDNF that lead to impaired plasticity and development of dyskinesia.

7. Summary and conclusions

In this thesis, the role of neuronal nicotinic acetylcholine receptors in mouse models of Parkinson's disease and levodopa-induced dyskinesia was investigated. The main findings and conclusions of the studies can be summarized as follows:

1. Hemiparkinsonian mice lacking α5* nAChRs showed attenuated dopaminergic neurodegeneration in the medial substantia nigra, attenuated amphetamine-induced rotational behavior, and less severe LID. Reduced dopamine transporter activity was observed in intact α5-knockout mice. These findings may be of significant relevance to human Parkinson's disease, and the potential of α5* nAChRs as a novel drug target for the treatment of the disease warrants further study. The findings also more generally support the significant involvement of nAChRs in Parkinson's disease and their drug target potential.

2. LID was reduced by chronic nicotine treatment in drinking water in hemiparkinsonian mice suffering from partial dopaminergic denervation. This finding supports a role for nAChRs as drug targets for alleviating dyskinesia associated with the treatment of Parkinson's disease with levodopa. However, severe LID in hemiparkinsonian mice suffering from near-total dopaminergic denervation was not alleviated by treatment with nAChR agonists. These findings suggest that a partially intact nigrostriatal pathway may be essential for optimal nAChR-mediated antidyskinetic efficacy. Potential clinical treatment of LID with nAChR agonists may thus lose its effectiveness as the disease progresses.

3. Synaptosomal dopamine release experiments suggest that in conditions of partial denervation, dampening of dopaminergic activity *via* downregulation of striatal presynaptic α6β2* nAChRs may have a significant role in mediating the antidyskinetic effects of chronic nicotine treatment. In particular, presynaptic α4α6β2β3 nAChRs could be an important mediator and a possible drug target for clinical applications. α5* nAChRs may also be involved in nicotine's effects on LID.

4. BDNF protein levels of the lesioned striatum were correlated with LID severity, confirming previous results measuring *Bdnf* mRNA and representing further evidence for a link between BDNF and LID. A levodopa-induced elevation in corticostriatal BDNF of the lesioned hemisphere, reported in previous short-term studies, was not observed after a long-term levodopa treatment. The previously reported elevation may thus reflect early sensitization.

5. The methods of stereotactic surgery and postoperative care were significantly improved. Intensive postoperative care was found to increase wellbeing and dramatically reduce mortality, enabling acceptable survival also in mice subjected to near-total unilateral dopaminergic denervation.

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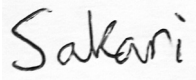

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